invitrogen™

ViraPower™ Promoterless Lentiviral Gateway® Kits

Using MultiSite Gateway® Technology to clone a promoter and gene of interest into a lentiviral vector for expression in dividing and non-dividing mammalian cells

Catalog nos. K591-10 and K5910-00

Revision date 31 October 2010

Manual part no. 25-0743

MAN0000465

User Manual

invitrogen™

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Kit Contents and Storage

Types of Kits

This manual is supplied with the following products.

Product	Catalog no.
ViraPower™ Promoterless Lentiviral Gateway® Vector Kit with MultiSite Gateway® Technology	K591–10
ViraPower™ Promoterless Lentiviral Gateway® Expression Kit with MultiSite Gateway® Technology	K5910–00

Kit Components

The ViraPower™ Promoterless Lentiviral Gateway® Kits include the following components. For a detailed description of the contents of each component, see pages v-vii. For a detailed description of the contents of the pENTR™5′-TOPO® TA Cloning® Kit and how to use the reagents supplied, see the pENTR™5′-TOPO® TA Cloning® Kit manual.

Component	Catalog no.	
	K591–10	**K5910–00**
pENTR™5′-TOPO® TA Cloning® Kit	√	√
ViraPower™ Lentiviral Vectors	√	√
One Shot® Stbl3™ Chemically Competent *E. coli*	√	√
LR Clonase™ II Plus Enzyme Mix		√
ViraPower™ Bsd Lentiviral Support Kit		√
293FT Cell Line		√

Continued on next page

Kit Contents and Storage, Continued

Shipping/Storage

The ViraPower™ Promoterless Lentiviral Gateway® Kits are shipped as described below. Upon receipt, store each item as detailed below.

Note: Catalog no. K591–10 includes the pENTR™5′-TOPO® TA Cloning® Kit, ViraPower™ Lentiviral Vectors, and One Shot® Stbl3™ Chemically Competent *E. coli* **only**.

Component	Shipping	Storage
ViraPower™ Lentiviral Vectors	Wet ice	–20°C
One Shot® Stbl3™ Chemically Competent *E. coli*	Dry ice	–80°C
LR Clonase™ Plus II Enzyme Mix	Dry ice	–80°C
ViraPower™ Bsd Lentiviral Support Kit	Wet ice	ViraPower™ Packaging Mix: –20°C Lipofectamine™ 2000: 4°C **(do not freeze)** Blasticidin: –20°C
293FT Cell Line	Dry ice	Liquid nitrogen
pENTR™5′-TOPO® TA Cloning® Kit	Dry ice	pENTR™5′-TOPO® Reagents: –20°C One Shot® TOP10 Chemically Competent *E. coli*: –80°C

ViraPower™ Lentiviral Vectors

The following vectors are included with the ViraPower™ Promoterless Lentiviral Gateway® Kits. All vectors are supplied in suspension detailed below. **Store the vectors at –20°C.**

Vector	Quantity	Contents
pLenti6/R4R2/V5-DEST	6 µg	40 µl of 150 ng/µl vector in 10 mM Tris-HCl, 1mM EDTA, pH 8.0
pLenti6/UbC/V5-GW/*lacZ*	10 µg	20 µl of 0.5 µg/µl control vector in 10 mM Tris-HCl, 1mM EDTA, pH 8.0
pENTR™5′/UbCp	10 µg	20 µl of 0.5 µg/µl vector in 10 mM Tris-HCl, 1mM EDTA, pH 8.0

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One Shot® Stbl3™ Chemically Competent *E. coli*

The following reagents are included with the One Shot® Stbl3™ Chemically Competent *E. coli* kit. Transformation efficiency is ≥1 × 10^8 cfu/µg plasmid DNA. **Store at –80°C.**

Product	Composition	Quantity
Stbl3™ Cells	--	21 × 50 µl
S.O.C. Medium	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	6 ml
pUC19 Control DNA	10 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	50 µl

Genotype of Stbl3™ *E. coli*

F^- *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ^- *leu mtl*-1

LR Clonase™ II Plus Enzyme Mix

The following reagents are supplied with the LR Clonase™ II Plus enzyme mix (Box 3). **Store at –20°C for up to 6 months. For long-term storage, store at –80°C.**

Product	Composition	Quantity
LR Clonase™ II Plus Enzyme Mix	Proprietary	40 µl
Proteinase K solution	2 µg/µl in: 10 mM Tris-HCl, pH 7.5, 20 mM $CaCl_2$, 50% glycerol	40 µl

Continued on next page

ViraPower™ Bsd Lentiviral Support Kit Contents

The ViraPower™ Bsd Lentiviral Support Kit includes the following reagents. **Store the Lipofectamine™ 2000 at 4°C and the ViraPower™ Packaging Mix and Blasticidin at –20°C.**

Important: Store Lipofectamine™ 2000 at 4°C. **Do not freeze.**

Product	Composition	Quantity
ViraPower™ Packaging Mix	Contains a mixture of the pLP1, pLP2, and pLP/VSVG plasmids, at 1 µg/µl in TE Buffer, pH 8.0	195 µg
Lipofectamine™ 2000	Proprietary	0.75 ml
Blasticidin	Powder	50 mg

293FT Cell Line

The 293FT Cell Line is supplied as one vial containing 3×10^6 frozen cells in 1 ml of Freezing Medium. **Upon receipt, store in liquid nitrogen.**

For instructions to thaw, culture, and maintain the 293FT Cell Line, see the 293FT Cell Line manual.

pENTR™5′-TOPO® TA Cloning® Kit

The ViraPower™ Promoterless Lentiviral Gateway® Kits include the pENTR™5′-TOPO® TA Cloning® Kit to facilitate production of an attL4 and attR1-flanked entry clone containing your eukaryotic promoter of interest. The pENTR™5′-TOPO® TA Cloning Kit contains:

- pENTR™5′-TOPO® Reagents
- One Shot® TOP10 Chemically Competent *E. coli*

Refer to the pENTR™5′-TOPO® TA Cloning® Kit manual for a detailed description of the reagents provided with the kit and instructions to produce the entry construct.

Accessory Products

Introduction

The products listed in this section may be used with the ViraPower™ Promoterless Lentiviral Gateway® Kits. For more information, refer to our website at www.invitrogen.com or contact **Technical Support** (page 56).

Accessory Products

Many of the reagents supplied in the ViraPower™ Promoterless Lentiviral Gateway® Kits as well as other products suitable for use with the kits are available separately from Invitrogen. Ordering information is provided below.

Product	Quantity	Catalog no.
pENTR™5′-TOPO® TA Cloning Kit	20 reactions	K591–20
One Shot® Stbl3™ Chemically Competent *E. coli*	20 × 50 µl	C7373–03
PureLink™ HiPure Plasmid Midiprep Kit	25 preps	K2100–04
S.N.A.P.™ Midiprep DNA Isolation Kit	20 reactions	K1910–01
Low DNA Mass Ladder	50 applications	10068–013
High DNA Mass Ladder	50 applications	10496–016
Gateway® LR Clonase™ II Plus Enzyme Mix	20 reactions 100 reactions	12538–020 12538–100
Ampicillin	5 g	Q100–16
ViraPower™ Bsd Lentiviral Support Kit	20 reactions	K4970–00
ViraPower™ Lentiviral Packaging Mix	60 reactions	K4975–00
293FT Cell Line	3×10^6 cells	R700–07
Library Efficiency® DB3.1™ Competent Cells	1 ml (5 × 0.2 ml)	11782–018
Lipofectamine™ 2000 Reagent	0.75 ml 1.5 ml	11668–027 11668–019
Opti-MEM® I Reduced Serum Medium	100 ml 500 ml	31985–062 31985–070
Blasticidin	50 mg	R210–01
β-gal Antiserum	50 µl*	R901–25

Detecting Recombinant Protein

You may detect expression of your recombinant protein using an antibody to the V5 epitope. Horseradish peroxidase (HRP) or alkaline phosphatase (AP)-conjugated antibodies allow one-step chemiluminescent or colorimetric detection. A fluorescein isothiocyanate (FITC)-conjugated antibody allows one-step detection in immunofluorescence experiments. The amount of antibody supplied is sufficient for 25 western blots.

Product	Quantity	Catalog No.
Anti-V5 Antibody	50 µl	R960–25
Anti-V5-HRP Antibody	50 µl	R961–25
Anti-V5-AP Antibody	125 µl	R962–25
Anti-V5-FITC Antibody	50 µl	R963–25

Introduction

Overview

Introduction

The ViraPower™ Promoterless Lentiviral Gateway® Expression System combines Invitrogen's ViraPower™ Lentiviral and MultiSite Gateway® technologies to facilitate lentiviral-based expression of a gene of interest from any promoter of choice in dividing or non-dividing mammalian cells. The System includes:

- The pENTR™5′-TOPO® TA Cloning Kit for production of an entry clone containing your eukaryotic promoter of interest. The pENTR™5′-TOPO® entry vector is adapted with MultiSite Gateway® Technology to facilitate transfer of the promoter sequence into the lentiviral expression plasmid.
- A promoterless pLenti6/R4R2/V5-DEST destination vector into which the promoter and gene of interest are transferred. This expression plasmid contains elements that allow packaging of the construct into virions and the Blasticidin resistance marker for selection of stably transduced cell lines.
- Components of the ViraPower™ Lentiviral System (Catalog no. K5910–00 only) for production of a replication-incompetent lentivirus that transiently or stably expresses the gene of interest in both dividing and non-dividing mammalian cells.

For more information about the ViraPower™ Lentiviral Technology and the MultiSite Gateway® Technology, see pages 6–7.

Advantages of the ViraPower™ Promoterless Lentiviral Gateway® Expression System

Use of the ViraPower™ Promoterless Lentiviral Gateway® Expression System to facilitate lentiviral-based expression of the gene of interest provides the following advantages:

- Allows production of a lentiviral construct that facilitates expression of a gene of interest under the control of a promoter of choice.
- Generates replication-incompetent lentivirus that effectively transduces both dividing and non-dividing mammalian cells, thus broadening the potential applications beyond those of traditional retroviral systems (Naldini, 1998).
- Efficiently delivers the gene of interest to mammalian cells in culture or *in vivo* (Dull *et al.*, 1998).
- Provides stable, long-term expression of a target gene beyond that offered by adenoviral-based systems (Dull *et al.*, 1998; Naldini *et al.*, 1996).
- Produces a pseudotyped virus with a broad host range (Yee *et al.*, 1994).
- The expression vector in the System is adapted with MultiSite Gateway® Technology for easy, simultaneous, recombination-based cloning of multiple DNA fragments in a defined order and orientation.
- Includes multiple features designed to enhance the biosafety of the system.

Continued on next page

Overview, Continued

ViraPower™ Lentiviral Technology

The ViraPower™ Lentiviral Technology facilitates highly efficient, *in vitro* or *in vivo* delivery of a target gene or RNA to dividing and non-dividing mammalian cells using a replication-incompetent lentivirus. Based on the lentikat™ system developed by Cell Genesys (Dull *et al.*, 1998), the ViraPower™ Lentiviral Technology possesses features which enhance its biosafety while allowing high-level expression in a wider range of cell types than traditional retroviral systems. The main components of the ViraPower™ Lentiviral Expression System include:

- A pLenti-based expression vector into which the DNA sequence (or sequences) are cloned. This vector contains elements required to allow packaging of the expression construct into virions and an antibiotic resistance marker to allow selection of stably transduced cell lines. For more information, see page 5.
- The ViraPower™ Packaging Mix, an optimized mixture of the three packaging plasmids required for production of the lentivirus.
- A 293FT producer cell line to facilitate optimal production of virus.

For more information about the ViraPower™ lentiviral components in this kit, see page 4. For more information about the biosafety features of the System, see page 8.

Purpose of this Manual

This manual provides an overview of the ViraPower™ Promoterless Lentiviral Gateway® Expression System and provides instructions and guidelines to:

1. Generate entry clones containing the promoter and gene of interest, one in pENTR™5′-TOPO® and the second in any Gateway® entry vector (guidelines only provided).
2. Use the pLenti6/R4R2/V5-DEST vector and two entry clones containing the promoter and gene of interest in a MultiSite Gateway® LR recombination reaction to generate an expression clone.
3. Cotransfect the pLenti6/R4R2/V5-DEST expression construct and the ViraPower™ Packaging Mix into the 293FT Cell Line to produce a lentiviral stock.
4. Titer the lentiviral stock.
5. Transduce the mammalian cell line of choice with the Lenti6/R4R2/V5-DEST lentiviral construct.
6. Assay for "transient" expression of your recombinant protein or generate a stably transduced cell line, if desired.

For details and instructions to generate the entry clone containing the promoter of interest, refer to the pENTR™5′-TOPO® TA Cloning Kit manual. For instructions to generate the entry clone containing the gene of interest, refer to the manual for the entry vector you select. For instructions to culture and maintain the 293FT producer cell line, refer to the 293FT Cell Line manual. The pENTR™5′-TOPO® TA Cloning® Kit and 293FT Cell Line manuals are supplied with Catalog no. K5910-00. All manuals are available for downloading from www.invitrogen.com or by contacting **Technical Support** (see page 56).

Continued on next page

Important

The ViraPower™ Promoterless Lentiviral Expression System is designed to help you create a lentivirus to deliver and express a gene of interest from a promoter of choice in mammalian cells. Although the system has been designed to help you express your recombinant protein of interest in the simplest, most direct fashion, use of the system is geared towards those users who are familiar with the principles of retrovirus biology and retroviral vectors. In addition, we highly recommend that users possess a working knowledge of:

- Viral and tissue culture techniques
- Gateway® Technology and site-specific recombination

For more information about these topics, refer to the following published reviews:

- Retrovirus biology and the retroviral replication cycle: see Buchschacher and Wong-Staal (2000) and Luciw (1996).
- Retroviral and lentiviral vectors: see Naldini (1999), Naldini (1998), and Yee (1999)
- Gateway® Technology and site-specific recombination: see Hartley *et al.* (2000) and Landy (1989)

Note

The One Shot® Stbl3™ Chemically Competent *E. coli*, LR Clonase™ II Plus Enzyme Mix, and Lipofectamine™ 2000 Reagent included in the ViraPower™ Promoterless Lentiviral Gateway® Expression System are available separately from Invitrogen and are each supplied with individual documentation detailing general use of the product. **For instructions to use these products specifically with the ViraPower™ Promoterless Lentiviral Gateway® Expression System, follow the recommended protocols in this manual.**

The ViraPower™ Promoterless Lentiviral Gateway® Expression System

Components of the ViraPower™ Promoterless Lentiviral Gateway® Expression System

The ViraPower™ Promoterless Lentiviral Gateway® Expression System facilitates highly efficient, lentiviral-based, *in vitro* or *in vivo* expression of a gene of interest under the control of a promoter of choice in dividing and non-dividing mammalian cells. The kit includes the following major components:

- The pENTR™5′-TOPO® TA Cloning Kit containing the pENTR™5′-TOPO® vector for production of an entry clone containing the promoter of interest. The vector is TOPO®-adapted and MultiSite Gateway®-adapted to allow TOPO® Cloning of a *Taq* polymerase-amplified PCR product encoding the promoter of interest and easy transfer of the promoter sequence into the pLenti6/R4R2/V5-DEST vector, respectively. For more information about the MultiSite Gateway® Technology, see page 6. For detailed information about the pENTR™5′-TOPO® vector and instructions to generate an entry clone, refer to the pENTR™5′-TOPO® TA Cloning® Kit manual.

  Important: To generate the pLenti6/R4R2/V5-DEST expression construct, you will also need to generate an entry clone containing your gene of interest. In this instance, you may use any standard Gateway® entry vector except pENTR™5′-TOPO®. For more information, see page 6.

- The pLenti6/R4R2/V5-DEST expression vector into which the promoter and gene of interest will be simultaneously cloned using MultiSite Gateway® Technology. The vector also contains the elements required for packaging of the expression construct into virions (*e.g.* 5′ and 3′ LTRs, ψ packaging signal) and the Blasticidin resistance marker to allow generation of stable cell lines. For more information about the pLenti6/R4R2/V5-DEST vector, see page 5.
- The ViraPower™ Packaging Mix that contains an optimized mix of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For more information about the packaging plasmids, see the **Appendix**, pages 50–55.
- An optimized 293FT producer cell line that stably expresses the SV40 large T antigen under the control of the human CMV promoter and facilitates optimal production of virus. For more information about the 293FT Cell Line, refer to the 293FT Cell Line manual.

After you have generated the pLenti6/R4R2/V5-DEST expression construct containing your promoter and gene of interest, you will cotransfect the plasmid and the ViraPower™ Packaging Mix into 293FT cells to produce a replication-incompetent lentiviral stock. This lentiviral stock may then be transduced into the mammalian cell line of interest to express your recombinant protein.

How Lentivirus Works

Once the lentivirus enters the target cell, the viral RNA is reverse-transcribed, actively imported into the nucleus (Lewis & Emerman, 1994; Naldini, 1999), and stably integrated into the host genome (Buchschacher & Wong-Staal, 2000; Luciw, 1996). After the lentiviral construct has integrated into the genome, you may assay for transient expression of your recombinant protein or use antibiotic selection to generate a stable cell line for long-term expression studies.

Continued on next page

VSV Envelope Glycoprotein

Most retroviral vectors are limited in their usefulness as gene delivery vehicles by their restricted tropism and generally low titers. In the ViraPower™ Promoterless Lentiviral Gateway® Expression System, this limitation has been overcome by use of the G glycoprotein gene from Vesicular Stomatitis Virus (VSV-G) as a pseudotyping envelope, thus allowing production of a high titer lentivirus with a significantly broadened host cell range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).

In vivo Gene Delivery

The ViraPower™ Promoterless Lentiviral Expression System is suitable for *in vivo* gene delivery applications. Many groups have successfully used lentiviral vectors to express a target gene in tissues including brain, retina, pancreas, muscle, liver, and skin (Gallichan *et al.*, 1998; Kafri *et al.*, 1997; Miyoshi *et al.*, 1997; Naldini, 1998; Pfeifer *et al.*, 2001; Pfeifer *et al.*, 2001; Takahashi *et al.*, 1999). For more information about target genes that have been successfully expressed *in vivo* using lentiviral-based vectors, refer to the references above as well as the following additional references (Baek *et al.*, 2001; Dull *et al.*, 1998; Lois *et al.*, 2002; Park & Kay, 2001; Peng *et al.*, 2001).

Features of the pLenti6/R4R2/V5-DEST Vector

The pLenti6/R4R2/V5-DEST vector contain the following elements:

- Rous Sarcoma Virus (RSV) enhancer/promoter for Tat-independent production of viral mRNA in the producer cell line (Dull *et al.*, 1998)
- Modified HIV-1 5′ and 3′ Long Terminal Repeats (LTR) for viral packaging and reverse transcription of the viral mRNA (Dull *et al.*, 1998; Luciw, 1996)

  Note: The U3 region of the 3′ LTR is deleted (ΔU3) and facilitates self-inactivation of the 5′ LTR after transduction to enhance the biosafety of the vector (Dull *et al.*, 1998)
- HIV-1 psi (Ψ) packaging sequence for viral packaging (Luciw, 1996)
- HIV Rev response element (RRE) for Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989)
- Two recombination sites, *att*R4 and *att*R2 for recombinational cloning of the promoter and gene of interest from two separate entry clones
- The *ccd*B gene located between the *att*R sites for negative selection
- Chloramphenicol resistance gene (Cm^R) located between the two *att*R sites for counterselection
- C-terminal V5 epitope for detection of the recombinant protein of interest (Southern *et al.*, 1991)
- Blasticidin resistance gene for selection in *E. coli* and mammalian cells (Izumi *et al.*, 1991; Kimura *et al.*, 1994; Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965)
- Ampicillin resistance gene for selection in *E. coli*
- pUC origin for high-copy replication of the plasmid in *E. coli*

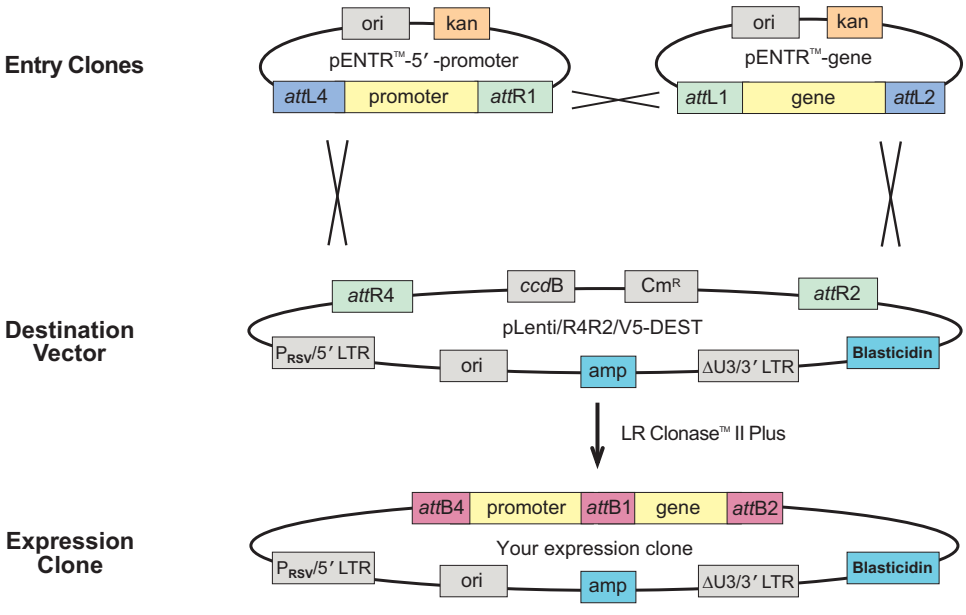
The MultiSite Gateway® Technology

The MultiSite Gateway® Technology

Gateway® Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to transfer a single DNA sequence of interest into multiple vector systems. The MultiSite Gateway® Technology uses modifications of the Gateway® Technology to allow simultaneous cloning of multiple DNA fragments in a defined order and orientation to create an expression construct. In the ViraPower™ Promoterless Lentiviral Gateway® Expression System, the MultiSite Gateway® Technology facilitates recombinational cloning of two DNA fragments encoding a promoter and gene of choice into the pLenti6/R4R2/V5-DEST lentiviral destination vector. To generate your lentiviral expression clone, you will:

1. TOPO® Clone the promoter of choice into the pENTR™5′-TOPO® vector containing *att*L4 and *att*R1 recombination sites to create a pENTR™5′-promoter entry clone. The pENTR™5′-TOPO® vector and manual are included in this kit.
2. Clone the gene of interest into any standard Gateway® entry vector containing *att*L1 and *att*L2 recombination sites to create a pENTR™-gene entry clone. For information about the Gateway® entry vectors available, see page 12.
3. Use the two entry clones in a single MultiSite Gateway® LR recombination reaction with the pLenti6/R4R2/V5-DEST vector containing *att*R4 and *att*R2 recombination sites to create your expression clone of interest (see the diagram below). For more information about pLenti6/R4R2/V5-DEST, see pages 5 and 46-47.

For more information about the Gateway® Technology, refer to the Gateway® Technology manual, available from www.invitrogen.com or by contacting **Technical Support** (see page 56).



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The MultiSite Gateway® Technology, Continued

att Sites

In the Gateway® Technology, recombinational cloning is mediated via optimized *att* sites. To accommodate simultaneous recombinational cloning of multiple DNA fragments in the MultiSite Gateway® Technology, these *att* sites have been further modified and optimized. Modifications include alterations to both the sequence and length of the *att* sites, resulting in the creation of "new" *att* sites exhibiting enhanced specificities and the improved efficiency required to permit cloning of multiple DNA fragments in a single reaction. In the ViraPower™ Promoterless Lentiviral Gateway® Expression System, the entry and destination vectors contain the following *att* sites (see the figure on the previous page):

- pENTR™ 5′-TOPO® containing your promoter of interest: *att*L4 and *att*R1
- Entry vector containing your gene of interest: *att*L1 and *att*L2
- pLenti6/R4R2/V5-DEST lentiviral destination vector: *att*R4 and *att*R2

To facilitate proper generation of a lentiviral expression construct, only this combination of entry clones and destination vector may be used in the MultiSite Gateway® LR recombination reaction.

Important

Note that the *att* sites used in MultiSite Gateway®-adapted vectors have been optimized to improve specificity and efficiency of the MultiSite Gateway® LR recombination reaction, and may vary in size and sequence from those used in the Gateway® Technology.

LR Clonase™ II Plus Enzyme Mix

The MultiSite Gateway® LR recombination reaction is catalyzed by LR Clonase™ II Plus enzyme mix. LR Clonase™ II Plus enzyme mix facilitates efficient recombinational cloning of multiple DNA fragments, but is also suitable for use in standard Gateway® LR reactions. Note, however, that standard LR Clonase™ enzyme mix **is not suitable** for use in the MultiSite Gateway® LR recombination reaction.

Note

Recombination between *att*R and *att*L sites generates *att*B sites (see figure on the previous page) in the lentiviral expression vector. We have shown that the presence of *att*B sites within the expression cassette does not affect gene expression.

Biosafety Features of the System

Introduction

The lentiviral and packaging vectors supplied in the ViraPower™ Promoterless Lentiviral Gateway® Expression System are third-generation vectors based on lentiviral vectors developed by Dull *et al.*, 1998 . This third-generation HIV-1-based lentiviral system includes a significant number of safety features designed to enhance its biosafety and to minimize its relation to the wild-type, human HIV-1 virus. These safety features are described below.

Biosafety Features of the ViraPower™ Promoterless Lentiviral System

The ViraPower™ Promoterless Lentiviral Gateway® Expression System includes the following key safety features:

- The pLenti6/R4R2/V5-DEST vector contains a deletion in the 3′ LTR (ΔU3) that does not affect generation of the viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Yee *et al.*, 1987; Yu *et al.*, 1986; Zufferey *et al.*, 1998). Once integrated into the transduced target cell, the lentiviral genome is no longer capable of producing packageable viral genome.
- The number of genes from HIV-1 that are used in the system has been reduced to three (*i.e. gag*, *pol*, and *rev*).
- The VSV-G gene from Vesicular Stomatitis Virus is used in place of the HIV-1 envelope (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
- Genes encoding the structural and other components required for packaging the viral genome are separated onto four plasmids (*i.e.* three packaging plasmids and pLenti6/R4R2/V5-DEST). All four plasmids have been engineered not to contain any regions of homology with each other to prevent undesirable recombination events that could lead to the generation of a replication-competent virus (Dull *et al.*, 1998).
- Although the three packaging plasmids allow expression *in trans* of proteins required to produce viral progeny (*e.g.* gal, pol, rev, env) in the 293FT producer cell line, none of them contain LTRs or the Ψ packaging sequence. This means that none of the HIV-1 structural genes are actually present in the packaged viral genome, and thus, are never expressed in the transduced target cell. No new replication-competent virus can be produced.
- The lentiviral particles produced in this system are replication-incompetent and only carry the gene of interest. No other viral species are produced.
- Expression of the *gag* and *pol* genes from pLP1 has been rendered Rev-dependent by virtue of the HIV-1 RRE in the gag/pol mRNA transcript. Addition of the RRE prevents gag and pol expression in the absence of Rev (Dull *et al.*, 1998).
- A constitutive promoter (RSV promoter) has been placed upstream of the 5′ LTR in the pLenti6/R4R2/V5-DEST vector to offset the requirement for Tat in the efficient production of viral RNA (Dull *et al.*, 1998).

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Biosafety Features of the System, Continued

Biosafety Level 2

Despite the inclusion of the safety features discussed on the previous page, the lentivirus produced with this System can still pose some biohazardous risk since it can transduce primary human cells. For this reason, **we highly recommend that you treat lentiviral stocks generated using this System as Biosafety Level 2 (BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.** Furthermore, exercise extra caution when creating lentivirus carrying potential harmful or toxic genes (*e.g.* activated oncogenes).

For more information about the BL-2 guidelines and lentivirus handling, refer to the document, "Biosafety in Microbiological and Biomedical Laboratories", 4th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at the following address:

http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

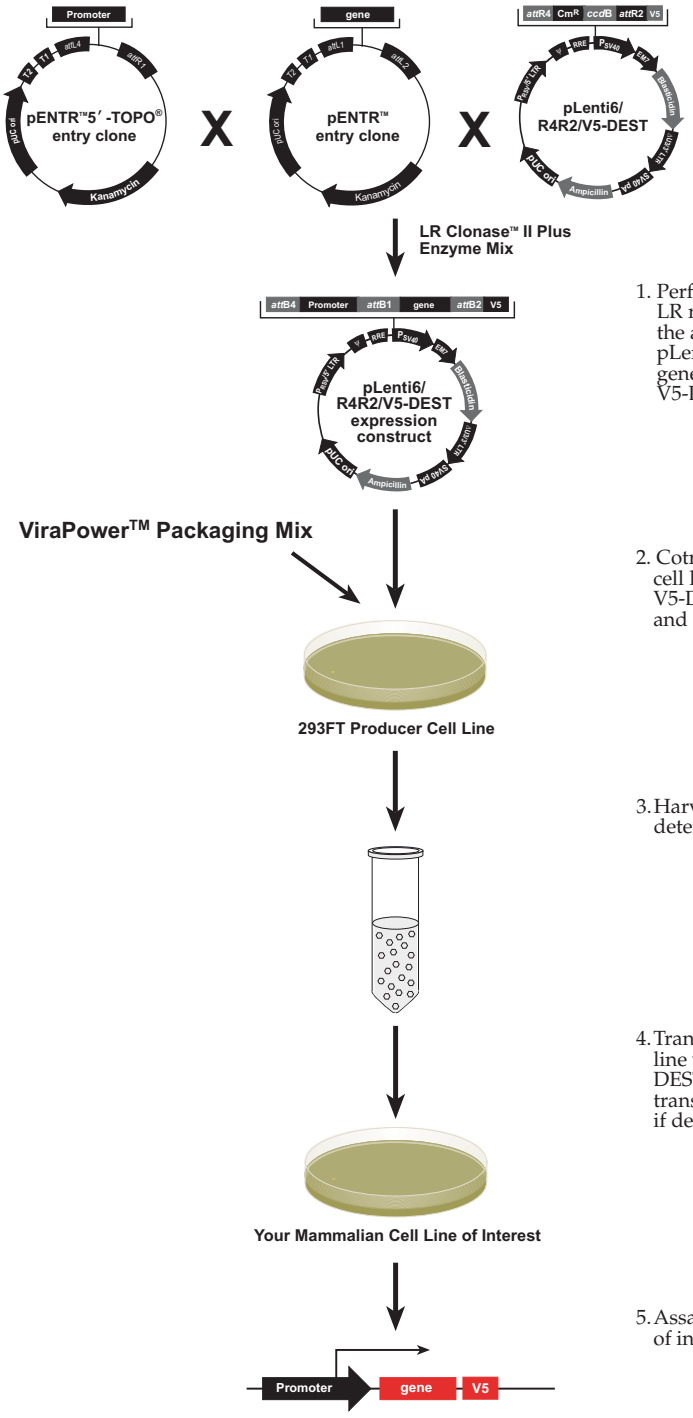
Important

Handle all lentiviruses in compliance with established institutional guidelines. Since safety requirements for use and handling of lentiviruses may vary at individual institutions, we recommend consulting the health and safety guidelines and/or safety officer(s) at your institution prior to use of the ViraPower™ Promoterless Lentiviral Gateway® Expression System.

Experimental Outline

Flow Chart

The diagram below describes the general steps required to express a gene of interest under the control of your own promoter using the ViraPower™ Promoterless Lentiviral Gateway® Expression System.



1. Perform a MultiSite Gateway® LR recombination reaction between the appropriate entry clones and pLenti6/R4R2/V5-DEST to generate the pLenti6/R4R2/V5-DEST expression construct.
2. Cotransfect the 293FT producer cell line with your pLenti6/R4R2/V5-DEST expression construct and the optimized Packaging Mix.
3. Harvest viral supernatant and determine the titer.
4. Transduce your mammalian cell line with the pLenti6/R4R2/V5-DEST lentivirus. Select for stably transduced cells using Blasticidin, if desired.
5. Assay for recombinant protein of interest.

Methods

Generating Entry Clones

Introduction

Before you can generate an expression construct in pLenti6/R4R2/V5-DEST, you will first need to generate the following entry clones:

- **An *att*L4 and *att*R1-flanked entry clone containing your eukaryotic promoter of interest.** To generate this entry clone, you **must** use the pENTR™5′-TOPO® entry vector and the pENTR™5′-TOPO® TA Cloning® Kit supplied with the ViraPower™ Promoterless Lentiviral Gateway® Kits. See below for more information.
- **An *att*L1 and *att*L2-flanked entry clone containing your gene of interest.** To generate this entry clone, you may use any traditional Gateway® entry vector or obtain an Ultimate™ ORF Clone available from Invitrogen. See page 12 for more information.

General guidelines are provided in this section to help you generate the appropriate entry clones. For detailed instructions, refer to the manual for the entry vector you are using.

Important

When generating your entry clones, note that for efficient packaging to occur, pLenti6/R4R2/V5-DEST has a limited cloning size of 4.5 to 5 kb. That is, **the combined size of your promoter + gene of interest must not exceed 4.5 to 5 kb.** Inserts larger than 5 kb can reduce packaging efficiency, resulting in lower lentiviral titers.

Generating an Entry Clone Containing a Promoter of Interest

The pENTR™5′-TOPO® TA Cloning® Kit containing the pENTR™5′-TOPO® vector is supplied with the ViraPower™ Promoterless Lentiviral Gateway® Kits to facilitate generation of an *att*L4 and *att*R1-flanked entry clone containing your eukaryotic promoter of interest. Note that you **must** use the pENTR™5′-TOPO® vector in this application; other Gateway® entry vectors are not suitable. To generate an entry clone using pENTR™5′-TOPO®, you will:

1. Use *Taq* polymerase to produce a PCR product encoding your eukaryotic promoter of interest.
2. TOPO® Clone the PCR product into pENTR™5′-TOPO® using a 5-minute bench-top ligation.
3. Transform the TOPO® Cloning reaction into chemically competent *E. coli* supplied with the kit and select for entry clones.

For instructions and protocols, refer to the pENTR™5′-TOPO® TA Cloning® Kit manual. This manual is supplied with the ViraPower™ Promoterless Lentiviral Gateway® Kits, but is also available for downloading from www.invitrogen.com or by contacting **Technical Support** (see page 56).

Note: The pENTR™5′-TOPO® TA Cloning® kit is also available separately from Invitrogen (page viii).

Continued on next page

Generating Entry Clones, Continued

Promoter Sequence Considerations

Consider the following when cloning your eukaryotic promoter sequence:

- Make sure that your DNA fragment contains all promoter and enhancer sequences (*e.g.* TATA box, transcription factor binding sites) necessary to regulate expression of the downstream gene of interest (following MultiSite Gateway® LR recombination).
- Make sure that your promoter sequence contains a transcription initiation site.
- Make sure that your promoter sequence does **not** contain an ATG translation initiation codon.

Generating an Entry Clone Containing a Gene of Interest

To generate an *att*L1 and *att*L2-flanked entry clone containing the gene of interest, you may use any Gateway® entry vector available from Invitrogen except pENTR™5′-TOPO®. For fast and easy generation of an entry clone using TOPO® Cloning, we recommend using the pENTR™/D-TOPO® entry vector. Other TOPO®-adapted entry vectors are available (see table below). See the next page for recommendations to produce the entry clone.

Once you have selected an entry vector, refer to the manual for the specific entry vector you are using for instructions to construct an entry clone. All entry vector manuals are available for downloading from www.invitrogen.com or by contacting **Technical Support** (see page 56).

Entry Vector Kit	Catalog No.
pENTR™/D-TOPO® Cloning Kit	K2400–20
pENTR™/SD/D-TOPO® Cloning Kit	K2420–20
pCR®8/GW/TOPO® TA Cloning Kit*	
• with One Shot® TOP10 Chemically Competent E. coli	K2500–20
• with One Shot® Mach1™-T1R Chemically Competent E. coli	K2520–20

*When used in a MultiSite Gateway® LR recombination reaction with a pENTR™5′-TOPO® entry clone and pLenti6/R4R2/V5-DEST, entry clones generated in pCR®8/GW/TOPO® recombine less efficiently, resulting in slightly fewer total colonies. If you want to maximize the number of MultiSite Gateway® LR recombinants obtained, we suggest generating *att*L1 and *att*L2-containing entry clones in pENTR™/D-TOPO®.

If you wish to express a human gene of interest in pLenti6/R4R2/V5-DEST, you may want to use an Ultimate™ Human ORF (hORF) Clone available from Invitrogen. The Ultimate™ hORF Clones are fully sequenced clones provided in an *att*L1 and *att*L2-flanked Gateway® entry vector that is ready to use in a recombin-ation reaction with a pENTR™5′-TOPO® entry clone and the pLenti6/R4R2/V5-DEST vector. For more information about the Ultimate™ hORF Clones available, visit www.invitrogen.com/clones or contact **Technical Support** (see page 56).

Continued on next page

ORF Sequence Considerations

pLenti6/R4R2/V5-DEST allows fusion of your gene of interest to a C-terminal tag. When generating your entry clone, remember that your gene of interest must:

- Contain a Kozak consensus sequence with an ATG initiation codon for proper initiation of translation (Kozak, 1987; Kozak, 1990; Kozak, 1991). An example of a Kozak consensus sequence is provided below. Other sequences are possible, but the G or A at position –3 and the G at position +4 (shown in bold) illustrates the most commonly occurring sequence with strong consensus. Replacing one of the two bases at these positions provides moderate consensus, while having neither results in weak consensus. The ATG initiation codon is shown underlined.

  (G/A)NN<u>ATG</u>**G**

- Be in frame with the C-terminal tag after recombination with pLenti6/R4R2/V5-DEST.
- **NOT** contain a stop codon.

Guidelines to Generate Expression Clones

Introduction

After you have generated separate entry clones containing your promoter and gene of interest, you will perform the MultiSite Gateway® LR recombination reaction to simultaneously transfer the two DNA fragments into the pLenti6/R4R2/V5-DEST vector to create an expression clone with the following structure:

***att*B4-promoter-*att*B1-gene of interest-*att*B2**

To ensure that you obtain the best possible results, we recommend that you read this section and the sections entitled **Performing the MultiSite Gateway® LR Recombination Reaction** (pages 17–22) and **Transforming One Shot® Stbl3™ Competent *E. coli*** (pages 21–23) before beginning.

Experimental Outline

To generate an expression clone, you will:

1. Perform a MultiSite Gateway® LR recombination reaction using the appropriate entry clones and pLenti6/R4R2/V5-DEST (see below).
2. Transform the reaction mixture into a suitable *E. coli* host.
3. Select for expression clones (see page 16 for an illustration of the recombination region of expression clones in pLenti6/R4R2/V5-DEST).

Substrates for the MultiSite Gateway® LR Recombination Reaction

To perform the two-fragment MultiSite Gateway® LR recombination reaction, you **must** have the substrates listed below.

- *att*L4 and *att*R1-flanked entry clone containing the promoter of interest
- *att*L1 and *att*L2-flanked entry clone containing the gene of interest
- *att*R4 and *att*R2-flanked pLenti6/R4R2/V5-DEST vector

Keep in mind the following:

- You **cannot** successfully create a two-fragment expression clone using the MultiSite Gateway® LR recombination reaction if you have any combination of *att*-flanked entry clones other than the ones listed above.
- You **must** use the pLenti6/R4R2/V5-DEST destination vector for this reaction. Other pLenti-based destination vectors or Gateway® destination vectors **cannot** be used.

Important

For optimal results, we recommend performing the MultiSite Gateway® LR recombination reaction using:

- Supercoiled entry clones
- Supercoiled pLenti6/R4R2/V5-DEST

Continued on next page

Guidelines to Generate Expression Clones, Continued

Plasmid Preparation

Once you have generated your entry clones, prepare purified plasmid DNA from each entry clone to use in the MultiSite Gateway® LR recombination reaction. You may use any method of choice to isolate plasmid DNA. We recommend using Invitrogen's PureLink™ HiPure Plasmid Midiprep Kit (page viii). DNA preparations are **not recommended** for MultiSite Gateway® cloning reactions. DNA cannot be quantitated by UV absorbance due to contaminating RNA and nucleotides, estimate concentration by gel electrophoresis (*e.g.*, DNA Mass Ladder, Cat. no. 10068–013 or 10496–016).

Resuspend the purified plasmid DNA in sterile water or TE Buffer, pH 8.0 to a final concentration of 150 ng/µl.

Vector Information

The pLenti6/R4R2/V5-DEST destination vector is supplied as 6 µg of supercoiled plasmid in 40 µl of 150 ng/µl vector in 10 mM Tris-HCL, 1mM EDTA, pH 8.0

Propagating the Destination Vector

If you wish to propagate and maintain the pLenti6/R4R2/V5-DEST vector, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (Catalog no. A10460) from Invitrogen. The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to *ccdB* effects and can support the propagation of plasmids containing the *ccd*B gene.

Note: Do not use general *E. coli* cloning strains including Stbl3™, TOP10 or DH5a™ for propagation and maintenance as these strains are sensitive to CcdB effects.

Guidelines to Propagate the Destination Vector

Follow the guidelines below when using DB3.1™ *E. coli* to propagate the pLenti6/R4R2/V5-DEST plasmid:

- To maintain integrity of the vector, select for transformants in media containing 50–100 µg/ml ampicillin and 15–30 µg/ml chloramphenicol.
- Due to the potential for rearrangement of lentiviral vectors caused by recombination between the 5′ and 3′ LTRs (*i.e.* unwanted recombinants), we recommend analyzing transformants to verify the integrity of the destination vector before proceeding.
- When propagating transformants, culture bacteria in LB media. **Do not use** "richer" bacterial medias as these media tend to give rise to a greater number of unwanted recombinants.

Continued on next page

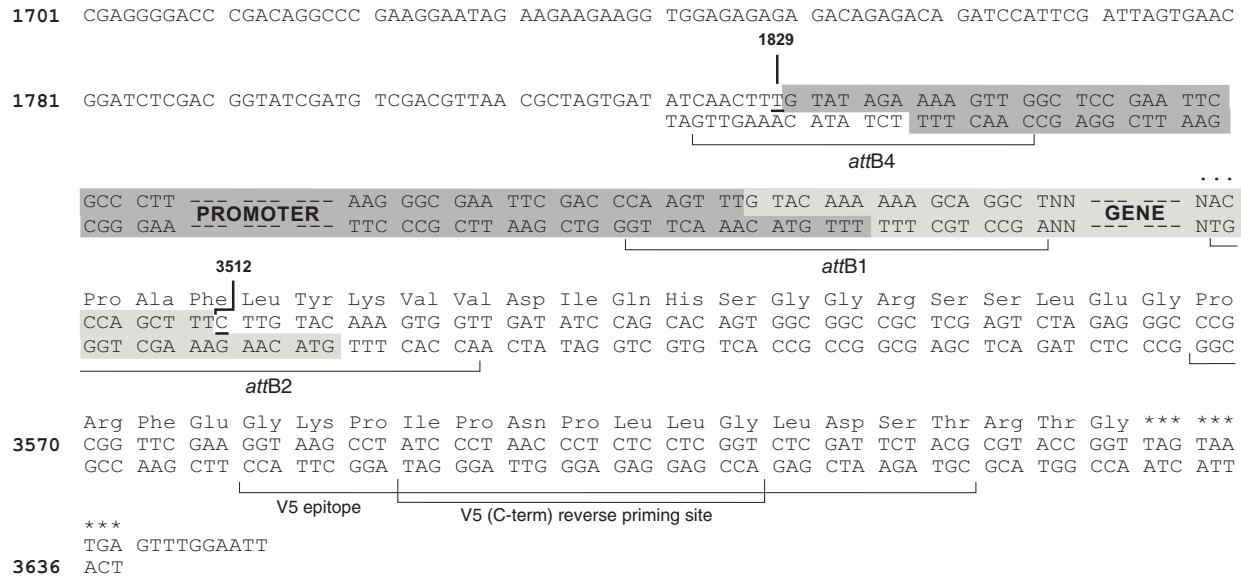
Guidelines to Generate Expression Clones, Continued

Recombination Region of the Expression Clone

The recombination region of the expression clone resulting from *att*L4-promoter-*att*R1 entry clone × *att*L1-gene-*att*L2 entry clone × pLenti6/R4R2/V5-DEST is shown below.

Features of the Recombination Region:

- Shaded regions correspond to those DNA sequences transferred from the two entry clones into the pLenti6/R4R2/V5-DEST vector by recombination. Note that the sequences comprising the *att*B1 site are entirely supplied by the entry clones. Non-shaded regions are derived from the pLenti6/R4R2/V5-DEST vector.
- Bases 1829 and 3512 of the pLenti6/R4R2/V5-DEST sequence are marked.


1701 CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC
1829
1781 GGATCTCGAC GGTATCGATG TCGACGTTAA CGCTAGTGAT ATCAACTTTG TAT AGA AAA GTT GGC TCC GAA TTC
TAGTTGAAAC ATA TCT TTT CAA CCG AGG CTT AAG
attB4
GCC CTT ___ ___ ___ AAG GGC GAA TTC GAC CCA AGT TTG TAC AAA AAA GCA GGC TNN ___ ___ NAC
CGG GAA ___ ___ ___ TTC CCG CTT AAG CTG GGT TCA AAC ATG TTT TTT CGT CCG ANN ___ ___ NTG
PROMOTER
GENE
3512
attB1
Pro Ala Phe Leu Tyr Lys Val Val Asp Ile Gln His Ser Gly Gly Arg Ser Ser Leu Glu Gly Pro
CCA GCT TTC TTG TAC AAA GTG GTT GAT ATC CAG CAC AGT GGC GGC CGC TCG AGT CTA GAG GGC CCG
GGT CGA AAG AAC ATG TTT CAC CAA CTA TAG GTC GTG TCA CCG CCG GCG AGC TCA GAT CTC CCG GGC
attB2
Arg Phe Glu Gly Lys Pro Ile Pro Asn Pro Leu Leu Gly Leu Asp Ser Thr Arg Thr Gly *** ***
3570 CGG TTC GAA GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG CGT ACC GGT TAG TAA
GCC AAG CTT CCA TTC GGA TAG GGA TTG GGA GAG GAG CCA GAG CTA AGA TGC GCA TGG CCA ATC ATT
V5 epitope
V5 (C-term) reverse priming site
***
TGA GTTTGGAATT
3636 ACT

Performing the MultiSite Gateway® LR Recombination Reaction

Important

A new enzyme (LR Clonase II Plus) is supplied in this kit, and the MultiSite Gateway® LR recombination reaction protocol has been changed. Follow the protocol below carefully.

Introduction

Follow the guidelines and instructions in this section to perform the MultiSite Gateway® LR recombination reaction using the appropriate entry clones and the pLenti6/R4R2/V5-DEST vector. We recommend including a negative control (no LR Clonase™ II Plus) in your experiment to help you evaluate your results.

Determining How Much DNA to Use in the Reaction

For optimal efficiency, we recommend using the following amounts of plasmid DNA (*i.e.* entry clones and destination vector) in a 10 µl MultiSite Gateway® LR recombination reaction:

- An equimolar amount of each plasmid.
- 10 fmoles of **each** entry clone and 20 fmoles of pLenti6/R4R2/V5-DEST is recommended.

For a formula to convert fmoles of DNA to nanograms (ng) and an example, see below.

Converting Femtomoles (fmoles) to Nanograms (ng)

Use the following formula to convert femtomoles (fmoles) of DNA to nanograms (ng) of DNA:

$$\text{ng} = (\text{x fmoles})(\text{N})(\frac{660\,\text{fg}}{\text{fmoles}})(\frac{1\,\text{ng}}{10^6\,\text{fg}})$$

where × is the number of fmoles and N is the size of the DNA in bp. For an example, see below.

Example of fmoles to ng Conversion

In this example, you need to use 50 fmoles of an *att*B PCR product in the BP reaction. The *att*B PCR product is 2.5 kb in size. Calculate the amount of *att*B PCR product required for the reaction (in ng) by using the equation above:

$$(50\,\text{fmoles})(2500\,\text{bp})(\frac{660\,\text{fg}}{\text{fmoles}})(\frac{1\,\text{ng}}{10^6\,\text{fg}}) = 82.5\,\text{ng of PCR product required}$$

Recommended *E. coli* Host

For optimal results, we recommend using Stbl3™ *E. coli* for transformation as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats. One Shot® Stbl3™ Chemically Competent *E. coli* are included in the kit for transformation. For instructions, see **Transforming One Shot® Stbl3™ Competent *E. coli***, page 21. Note that transformants containing unwanted recombinants (see **Note** below) are obtained less frequently when Stbl3™ *E. coli* are used for transformation.

Note

You may transform the LR recombination reaction into other *rec*A, *end*A *E. coli* strains including TOP10 and DH5α™, if desired. Note however, that these strains are not as well-suited for cloning unstable DNA, and may give rise to a low percentage (< 5%) of transformants containing unwanted recombinants (*i.e.* plasmids where recombination has occurred between the 5′ and 3′ LTRs) when selected on plates containing only ampicillin. If you wish to use TOP10 or DH5α™ cells for transformation, follow the guidelines below to reduce the frequency of obtaining unwanted recombinants:

- **Select for transformants using 100 µg/ml ampicillin and 50 µg/ml Blasticidin.** Note that transformed *E. coli* grow more slowly in LB media containing two selection agents, and may require slightly longer incubation times to obtain visible colonies. For a recipe to prepare LB agar plates containing ampicillin and Blasticidin, see page 44. For more information about Blasticidin, see page 45.
- **Select small colonies for analysis** as transformants containing a plasmid that has recombined between the 5′ and 3′ LTRs (*i.e.* unwanted recombinants) generally give rise to larger colonies than those containing an intact plasmid.

Important

Do not transform the MultiSite Gateway® LR recombination reaction into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Positive Control Entry Clone

The pENTR™5′-UbCp plasmid is included with the kit for use as a positive control for the MultiSite Gateway® LR recombination reaction, and is a pENTR™5′ entry clone containing the human UbC promoter. You may use this entry clone together with any *att*L1 and *att*L2-flanked entry clone in your MultiSite Gateway® LR recombination reaction to verify the efficiency of the reaction. For a map of pENTR™5′-UbCp, see the **Appendix**, page 48.

Performing the MultiSite Gateway® LR Recombination Reaction, Continued

Materials Needed

You will need the following items:

- 10 fmoles purified plasmid DNA of your *att*L4 and *att*R1-flanked entry clone
- 10 fmoles purified plasmid DNA of your *att*L1 and *att*L2-flanked entry clone
- 10 fmoles control plasmid pENTR™5′/UbCp (if desired)
- 20 fmoles pLenti6/R4R2/V5-DEST vector
- LR Clonase™ II Plus enzyme mix (supplied with Catalog no. K5910–00; keep at –80°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase™ II Plus enzyme mix; thaw and keep on ice until use)
- Sterile 0.5 ml microcentrifuge tubes

Important

You must use LR Clonase™ II Plus enzyme mix for this application. Do not use standard LR Clonase™ or LR Clonase™ II enzyme mix.

LR Clonase™ II Plus enzyme mix is supplied with Catalog no. K5910–00, but is also available separately from Invitrogen. See page viii for ordering information.

Continued on next page

Performing the MultiSite Gateway® LR Recombination Reaction, Continued

Setting Up the MultiSite Gateway® LR Recombination Reaction

Follow this procedure to perform the MultiSite Gateway® LR recombination reaction between your entry clones and the pLenti6/R4R2/V5-DEST vector. If you want to include a negative control, set up a separate reaction in which you omit the LR Clonase™ II Plus enzyme mix.

1. Add the following components to 0.5 ml microcentrifuge tubes at room temperature and mix.

Component	Sample	Negative Control	Positive Control
pENTR™5′-promoter entry clone (10 fmol)	1–7 µl	1–7 µl	--
pENTR™-gene entry clone (10 fmol)	--	--	1–6 µl
pENTR™5′/UbCp (10 fmol)	--	--	1 µl
pLenti6/R4R2/V5-DEST (20 fmol)	1 µl	1 µl	1 µl
TE Buffer, pH 8.0	to 8 µl	to 8 µl	to 8 µl

2. Remove the LR Clonase™ II Plus enzyme mix from –80°C and thaw on ice (~2 minutes).
3. Vortex the LR Clonase™ II Plus enzyme mix briefly twice (2 seconds each time).
4. Add 2 µl of LR Clonase™ II Plus enzyme mix to the sample and positive control vials. **Do not** add LR Clonase™ to the negative control vial. Mix well by pipetting up and down.

   Reminder: Return LR Clonase™ II Plus enzyme mix to –80°C immediately after use.
5. Incubate the reaction at room temperature (20–25°C) from 16–24 hours.
6. Add 1 µl of the Proteinase K solution to each reaction. Incubate for 10 minutes at 37°C.
7. Proceed to **Transforming One Shot® Stbl3™ Competent *E. coli***, next page.

   Note: You may store the MultiSite Gateway® LR reaction at –20°C for up to 1 week before transformation, if desired.

Transforming One Shot® Stbl3™ Competent *E. coli*

Introduction

Follow the instructions in this section to transform the MultiSite Gateway® LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli* included with the kit. The transformation efficiency of One Shot® Stbl3™ Chemically Competent *E. coli* is ≥1 × 10^8 cfu/µg plasmid DNA.

Materials Needed

You will need the following items:

- MultiSite Gateway® LR recombination reaction (from Step 7, previous page)
- LB Medium (if performing the pUC19 control transformation)
- LB plates containing 100 µg/ml ampicillin (two for each transformation; warm at 37°C for 30 minutes before use)
- 42°C water bath
- 37°C shaking and non-shaking incubator

Materials supplied with kit

- One Shot® Stbl3™ Chemically Competent *E. coli* (one vial per transformation; thaw on ice immediately before use)
- S.O.C. Medium (room temperature)
- pUC19 positive control (if desired to verify the transformation efficiency)

One Shot® Stbl3™ Transformation Procedure

Use this procedure to transform the MultiSite Gateway® LR recombination reaction into One Shot® Stbl3™ Chemically Competent *E. coli*.

1. Thaw, on ice, one vial of One Shot® Stbl3™ chemically competent cells for each transformation.
2. Add 2 µl of the MultiSite Gateway® LR recombination reaction (from Step 7, previous page) into a vial of One Shot® Stbl3™ cells and mix gently. **Do not mix by pipetting up and down.** For the pUC19 control, add 10 pg (1 µl) of DNA into a separate vial of One Shot® cells and mix gently.
3. Incubate the vial(s) on ice for 30 minutes.
4. Heat-shock the cells for 45 seconds at 42°C without shaking.
5. Remove the vial(s) from the 42°C water bath and place on ice for 2 minutes.
6. Add 250 µl of pre-warmed S.O.C. Medium to each vial.
7. Cap the vial(s) tightly and shake horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.
8. Spread 25–100 µl of the transformation mix on a pre-warmed selective plate and incubate overnight at 37°C. We recommend plating two different volumes to ensure that at least one plate will have well-spaced colonies. For the pUC19 control, dilute the transformation mix 1:10 into LB Medium (*e.g.* add 100 µl of the transformation mix to 900 µl of LB Medium) and plate 25–100 µl.
9. Store the remaining transformation mix at 4°C. Plate out additional cells the next day, if desired.

Continued on next page

What You Should See

If you use *E. coli* cells with a transformation efficiency of 1×10^9 cfu/µg, the MultiSite Gateway® LR reaction should give approximately 1,000 to 5,000 colonies if the entire reaction is transformed and plated.

Note: If you performed the MultiSite Gateway® LR recombination reaction using a pCR®8/GW/TOPO® entry clone, fewer total colonies may be obtained.

Analyzing Positive Clones

1. Pick 5 colonies and culture them overnight in LB medium containing 100 µg/ml ampicillin.
2. Isolate plasmid DNA using your method of choice.
3. Analyze the plasmids by restriction analysis to confirm the presence and orientation of your inserts (promoter + gene) as well as the integrity of the vector.

Confirming the Expression Clone

The *ccd*B gene mutates at a very low frequency, resulting in a very low number of false positives. True expression clones will be chloramphenicol-sensitive **and** ampicillin- and Blasticidin-resistant. Transformants containing a plasmid with a mutated *ccdB* gene will be chloramphenicol-, ampicillin-, and Blasticidin-resistant. To check your putative expression clone, test for growth on LB plates containing 30 µg/ml chloramphenicol. A true expression clone should not grow in the presence of chloramphenicol.

Sequencing

Sequencing the expression construct is not required as transfer of the promoter and gene of interest from the entry vectors into the pLenti6/R4R2/V5-DEST vector preserves the orientation and reading frame. However, if you wish to confirm that your gene of interest is in frame with the C-terminal tag in pLenti6/R4R2/V5-DEST, you may sequence your expression construct. We recommend using the following primer for sequencing. Refer to the diagram on page 16 for the location of the primer binding site in the vector.

Note: For your convenience, Invitrogen has a custom primer synthesis service. For more information, visit our website at www.invitrogen.com or contact **Technical Support** (see page 56).

Primer	Sequence
V5 (C-term) reverse primer	5′-ACCGAGGAGAGGGTTAGGGAT-3′

Maintaining the Expression Clone

Once you have generated your expression clone, maintain and propagate the plasmid in LB medium containing 100 µg/ml ampicillin. Addition of Blasticidin is not required.

Continued on next page

Verifying Expression of Recombinant Protein

Optional: Before proceeding to generate a lentiviral stock of your pLenti6/R4R2/V5-DEST expression construct, you may verify that the construct expresses the gene of interest by transfecting the plasmid directly into mammalian cells and assaying for your recombinant protein, if desired. Follow the guidelines below:

- Use an easy-to-transfect, dividing mammalian cell line (*e.g.* HEK 293 or COS-7).
- Use a transfection reagent that allows high-efficiency transfection; we recommend using Lipofectamine™ 2000 Reagent (see page 26).

  Note: Lipofectamine™ 2000 is supplied with the ViraPower™ Promoterless Lentiviral Gateway® Expression System, but is also available separately from Invitrogen (see page viii for ordering information).
- Follow the manufacturer's instructions for the transfection reagent you are using to perform plasmid transfection. If you are using Lipofectamine™ 2000, follow the instructions included with the product.

Producing Lentivirus in 293FT Cells

Introduction

Once you have generated your pLenti6/R4R2/V5-DEST expression construct containing your promoter and gene of interest, you will cotransfect the expression construct and the optimized ViraPower™ Packaging Mix into 293FT cells to produce a lentiviral stock. This section provides protocols and instructions to generate a lentiviral stock.

Plasmid Preparation

Once you have generated your expression construct, you must isolate plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be clean and free from contamination with phenol and sodium chloride. Contami-nants may kill the cells, and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating plasmid DNA using the PureLink™ HiPure Plasmid Midiprep Kit or the S.N.A.P.™ Midiprep Kit (page viii).

Resuspend the purified pLenti6/R4R2/V5-DEST expression plasmid containing your promoter + gene of interest in sterile water or TE Buffer, pH 8.0 to a final concentration ranging from 0.1–3.0 µg/µl. You will need 3 µg of the expression plasmid for each transfection.

Important: Do not use mini-prep plasmid DNA for transfection.

Positive Control

The pLenti6/UbC/V5-GW/*lac*Z plasmid is included with the ViraPower™ Promoterless Lentiviral Gateway® kits for use as a positive control for lentivirus production and expression, and facilitates constitutive expression of β-galactosidase under the control of the human UbC promoter.

We recommend including the positive control vector in your cotransfection experiment to generate a control lentiviral stock. Once generated, use the control lentivirus to help you optimize expression conditions in your mammalian cell line of interest.

The pLenti6/UbC/V5-GW/*lac*Z control vector is supplied in suspension in TE Buffer, pH 8.0. You will need 3 µg of the plasmid for transfection. For a map of pLenti6/UbC/V5-GW/*lac*Z, see the **Appendix**, page 49.

Note: If you wish to propagate the pLenti6/R4R2/V5-DEST plasmid, use 10 ng of vector to transform a *rec*A, *end*A *E. coli* strain (*e.g.* Stbl3™). Select for transformants on LB agar plates containing 100 µg/ml ampicillin. If you use an *E. coli* strain other than Stbl3™ for transformation (*e.g.* TOP10 or DH5α), select for transformants on LB agar plates containing 100 µg/ml ampicillin **and** 50 µg/ml Blasticidin.

Continued on next page

Materials Needed

You will need the following items:

- pLenti6/R4R2/V5-DEST expression construct (0.1-3.0 µg/µl in sterile water or TE Buffer, pH 8.0)
- pLenti6/UbC/V5-GW/*lacZ* control vector (supplied with the kit)
- ViraPower™ Packaging Mix (supplied with Catalog no. K5910-00; resuspend in 195 µl of sterile water to a concentration of 1 µg/µl; see below for more information)
- 293FT cells cultured in the appropriate medium (*i.e.* D-MEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1% penicillin-streptomycin; see the next page for more information)
- Lipofectamine™ 2000 transfection reagent (supplied with Catalog no. K5910-00; store at 4°C and mix gently before use; see page 26 for more information)
- Opti-MEM® I Reduced Serum Medium (pre-warmed; see page 26 for more information)
- Fetal bovine serum (FBS)
- Complete growth medium containing sodium pyruvate (*i.e.* D-MEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin-streptomycin, and 1 mM MEM Sodium Pyruvate)

  Note: MEM Sodium Pyruvate provides an extra energy source for the cells and is available from Invitrogen as a 100 mM stock solution (page viii).
- Sterile, 10 cm tissue culture plates (one each for the lentiviral construct, positive control, and negative control)
- Sterile, tissue culture supplies
- 15 ml sterile, capped, conical tubes
- Cryovials

ViraPower™ Packaging Mix

The ViraPower™ Packaging Mix facilitates viral packaging of pLenti-based expression constructs following cotransfection into 293FT producer cells, and contains an optimized mixture of the pLP1, pLP2, pLP/VSVG plasmids. The amount of the Packaging Mix (195 µg) and Lipofectamine™ 2000 transfection reagent (0.75 ml) supplied in the ViraPower™ Promoterless Lentiviral Gateway® Expression System is sufficient to perform 20 cotransfections in 10 cm plates using the recommended protocol on page 28. For more information about the pLP1, pLP2, and pLP/VSVG plasmids, see the **Appendix**, pages 50–55.

Note: ViraPower™ Packaging Mix is available separately from Invitrogen (page viii) or as part of the ViraPower™ Lentiviral Support Kits (page viii).

Continued on next page

293FT Cell Line

The human 293FT Cell Line is supplied with the ViraPower™ Promoterless Lentiviral Gateway® Expression System to facilitate optimal lentivirus production (Naldini *et al.*, 1996). The 293FT Cell Line, a derivative of the 293F Cell Line, stably and constitutively expresses the SV40 large T antigen from pCMVSPORT6TAg.neo and **must** be maintained in medium containing Geneticin®. For more information about pCMVSPORT6TAg.neo and how to culture and maintain 293FT cells, refer to the 293FT Cell Line manual. This manual is supplied with the ViraPower™ Promoterless Lentiviral Gateway® Expression System, and is also available for downloading from our website at www.invitrogen.com or by contacting **Technical Support** (page 56).

Note: The 293FT Cell Line is available separately from Invitrogen (see page ix).

Important

The health of your 293FT cells at the time of transfection has a critical effect on the success of lentivirus production. Use of "unhealthy" cells can negatively affect the transfection efficiency, resulting in production of a low titer lentiviral stock. For optimal lentivirus production (*i.e.* producing lentiviral stocks with the expected titers), follow the guidelines below to culture 293FT cells before use in transfection:

- Make sure that cells are healthy and greater than 90% viable.
- Subculture and maintain cells as recommended in the 293FT Cell Line manual. **Do not allow cells to overgrow before passaging.**
- Use cells that have been subcultured for less than 20 passages.

Lipofectamine™ 2000

The Lipofectamine™ 2000 Reagent supplied with the ViraPower™ Promoterless Lentiviral Gateway® Expression System (Ciccarone *et al.*, 1999) is a proprietary, cationic lipid-based formulation suitable for the transfection of nucleic acids into eukaryotic cells. Using Lipofectamine™ 2000 to transfect 293FT cells offers the following advantages:

- Provides the highest transfection efficiency in 293FT cells
- DNA-Lipofectamine™ 2000 complexes can be added directly to cells in culture medium in the presence of serum
- Removal of complexes or medium change or addition following transfection is not required, although complexes can be removed after 4-6 hours without loss of activity

Note: Lipofectamine™ 2000 is available separately from Invitrogen or as part of the ViraPower™ Lentiviral Support Kits (see page viii for ordering information).

Opti-MEM® I

To facilitate optimal formation of DNA-Lipofectamine™ 2000 complexes, we recommend using Opti-MEM® I Reduced Serum Medium available from Invitrogen (see page viii for ordering information). For more information about Opti-MEM® I, visit our website at www.invitrogen.com or contact **Technical Support** (page 56).

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Recommended Transfection Conditions

We produce lentiviral stocks in 293FT cells using the following **optimized** transfection conditions below. The amount of lentivirus produced using these recommended conditions (at a titer of 1×10^5 to 1×10^7 transducing units (TU)/ml) is generally sufficient to transduce 1×10^6 to 1×10^8 cells at a multiplicity of infection (MOI) = 1.

Condition	Amount
Tissue culture plate size	10 cm (one per lentiviral construct)
Number of 293FT cells to transfect	6×10^6 cells (see **Important Note** on page 26 to prepare cells for transfection)
Amount of ViraPower™ Packaging Mix to use	9 µg (9 µl of 1 µg/µl stock)
Amount of pLenti-based expression plasmid to use	3 µg
Amount of Lipofectamine™ 2000 to use	36 µl

Note: You may produce lentiviral stocks using other tissue culture formats, but optimization will be necessary to obtain the expected titers.

The recommended procedure to cotransfect 293FT cells differs from the traditional Lipofectamine™ 2000 transfection procedure in that you will:

1. First prepare DNA-Lipofectamine™ 2000 complexes and add them to plates containing growth media, then
2. Add the 293FT cells to the media containing DNA-Lipofectamine™ 2000 complexes and allow the cells to attach and transfect overnight (see detailed procedure on the next page).

Using this procedure, we consistently obtain lentiviral stocks with titers that are **3- to 4-fold higher** than lentiviral stocks generated using the traditional Lipofectamine™ 2000 transfection procedure (*i.e.* plating cells first followed by transfection with DNA-Lipofectamine™ 2000 complexes). You may use the traditional Lipofectamine™ 2000 transfection procedure, if desired, but keep in mind that lower viral titers may be obtained (see **Alternative Transfection Procedure**, page 29).

Continued on next page

Transfection Procedure

Follow the procedure below to cotransfect 293FT cells. We recommend including a negative control (no DNA, no Lipofectamine™ 2000) in your experiment to help evaluate your results. You will need 6×10^6 293FT cells for each sample.

1. **For each transfection sample**, prepare DNA-Lipofectamine™ 2000 complexes as follows:
   a. In a sterile 5 ml tube, dilute 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti-based plasmid DNA (12 µg total) in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently.
   b. In a separate sterile 5 ml tube, mix Lipofectamine™ 2000 gently before use, then dilute 36 µl in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature.
   c. After the 5 minute incubation, combine the diluted DNA with the diluted Lipofectamine™ 2000. Mix gently.
   d. Incubate for 20 minutes at room temperature to allow the DNA-Lipofectamine™ 2000 complexes to form. The solution may appear cloudy, but this will not impede the transfection.
2. While DNA-lipid complexes are forming, trypsinize and count the 293FT cells. Resuspend the cells at a density of 1.2×10^6 cells/ml in growth medium or Opti-MEM® I Medium containing serum. **Do not include antibiotics in the medium.**
3. Add the DNA-Lipofectamine™ 2000 complexes to a 10 cm tissue culture plate containing 5 ml of growth medium or Opti-MEM® I Medium containing serum. **Do not include antibiotics in the medium.**
4. Add 5 ml of the 293FT cell suspension (6×10^6 total cells) to the plate containing media and DNA-Lipofectamine™ 2000 complexes. Mix gently by rocking the plate back and forth. Incubate cells overnight at 37°C in a CO_2 incubator.
5. The next day, remove the medium containing the DNA-Lipofectamine™ 2000 complexes and replace with complete culture medium containing sodium pyruvate (*i.e.* D-MEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM MEM Non-Essential Amino Acids, 1% penicillin/streptomycin, and 1 mM MEM Sodium Pyruvate).

   Note: Expression of the VSV G glycoprotein causes 293FT cells to fuse, resulting in the appearance of multinucleated syncytia. This morphological change is normal and does not affect production of the lentivirus.
6. Harvest virus-containing supernatants 48–72 hours post-transfection by removing medium to a 15 ml sterile, capped, conical tube. **Note:** Minimal differences in viral yield are observed whether supernatants are collected 48 or 72 hours post-transfection.

   Caution: Remember that you are working with infectious virus at this stage. Follow the recommended guidelines for working with BL-2 organisms (see page 9 for more information).
7. Centrifuge at 3000 rpm for 15 minutes at 4°C to pellet cell debris. Perform filtration step, if desired (see **Note** on the next page).
8. Pipet viral supernatants into cryovials in 1 ml aliquots. Store viral stocks at –80°C. Proceed to **Titering Your Lentiviral Stock**, page 30.

Continued on next page

Alternative Transfection Procedure

An alternative transfection procedure is provided below to cotransfect 293FT cells. Note that use of this procedure generally results in production of lentiviral stocks with a slightly lower titer than those produced when using the **Recommended Transfection Procedure**, previous page.

1. The day before transfection, plate the 293FT cells in a 10 cm tissue culture plate such that they will be 90–95% confluent on the day of transfection (*i.e.* 6×10^6 cells in 10 ml of growth medium containing serum).
2. On the day of transfection, remove the culture medium from the 293FT cells and replace with 5 ml of growth medium (or Opti-MEM® I Medium) containing serum. **Do not include antibiotics in the medium.**
3. Prepare DNA-Lipofectamine™ 2000 complexes as instructed in the **Recommended Transfection Procedure**, Step 1, previous page.
4. Add the DNA-Lipofectamine™ 2000 complexes drop-wise to each plate of cells. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37°C in a CO_2 incubator.
5. Follow Steps 5–8 as instructed in the **Recommended Transfection Procedure**, previous page.

Note

If you plan to use your lentiviral construct for *in vivo* applications, we recommend filtering your viral supernatant through a sterile, 0.45 µm low protein binding filter after the low-speed centrifugation step (see Step 7, previous page) to remove any remaining cellular debris. We recommend using Millex-HV 0.45 µm PVDF filters (Millipore, Catalog no. SLHVR25LS) for filtration.

If you wish to concentrate your viral stock to obtain a higher titer, perform the filtration step first before concentrating your viral stock.

Long-Term Storage

Place lentiviral stocks at –80°C for long-term storage. Repeated freezing and thawing is not recommended as it may result in loss of viral titer. When stored properly, viral stocks of an appropriate titer should be suitable for use for up to one year. After long-term storage, we recommend re-titering your viral stocks before transducing your mammalian cell line of interest.

Scaling Up Virus Production

It is possible to scale up the cotransfection experiment to produce a larger volume of lentivirus, if desired. For example, we have scaled up the cotransfection experiment from a 10 cm plate to a T-175 cm^2 flask and harvested up to 30 ml of viral supernatant. If you wish to scale up your cotransfection, remember that you will need to increase the number of cells plated and the amounts of DNA, Lipofectamine™ 2000, and medium used in proportion to the difference in surface area of the culture vessel.

Titering Your Lentiviral Stock

Introduction

Before proceeding to transduction and expression experiments, we highly recommend determining the titer of your lentiviral stock(s). While this procedure is not required for some applications, it is necessary if:

- You wish to control the number of integrated copies of the lentivirus.
- You wish to generate reproducible expression results.

Guidelines and protocols are provided in this section to titer your lentiviral stocks.

Experimental Outline

To determine the titer of a lentiviral stock, you will:

1. Prepare 10-fold serial dilutions of your lentiviral stock.
2. Transduce the different dilutions of lentivirus into the mammalian cell line of choice in the presence of Polybrene®.
3. Select for stably transduced cells using Blasticidin.
4. Stain and count the number of Blasticidin-resistant colonies in each dilution.

Factors Affecting Viral Titer

A number of factors can influence viral titers including:

- The size of your insert (promoter + gene) of interest. Titers will generally decrease as the size of the insert increases. The size of the wild-type HIV-1 genome is approximately 10 kb. Since the size of the elements required for expression from pLenti6/R4R2/V5-DEST total approximately 6 kb, the size of your insert (promoter + gene) should theoretically not exceed 4.5–5 kb for efficient packaging to occur.
- The characteristics of the cell line used for titering (see the next page for more information).
- The age of your lentiviral stock. Viral titers may decrease with long-term storage at –80°C. If your lentiviral stock has been stored for longer than 6 months, we recommend titering or re-titering your lentiviral stock prior to use.
- Number of freeze/thaw cycles. Viral titers can decrease as much as 10% with each freeze/thaw cycle.
- Improper storage of your lentiviral stock. Lentiviral stocks should be aliquotted and stored at –80°C (see page 29 for recommended storage conditions).

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Polybrene® is a registered trademark of Abbott Laboratories

Selecting a Cell Line

You may titer your lentiviral stock using any mammalian cell line of choice. Generally, we recommend using the same mammalian cell line to titer your lentiviral stock as you will use to perform your expression studies. However, in some instances, you may wish to use a different cell line to titer your lentivirus (*e.g.* if you are performing expression studies in a non-dividing cell line or a primary cell line). In these cases, we recommend that you choose a cell line with the following characteristics to titer your lentivirus:

- Grows as an adherent cell line
- Easy to handle
- Exhibits a doubling time in the range of 18–25 hours
- Non-migratory

We generally use the HT1080 human fibrosarcoma cell line (ATCC, Catalog no. CCL-121) for titering purposes.

Important: You may use other cell lines including HeLa and NIH/3T3 to titer your lentivirus. However, note that the titer obtained when using HeLa cells or NIH/3T3 cells is approximately **10-fold lower** than the titer obtained when using HT1080 cells.

Note

The titer of a lentiviral construct may vary depending on which cell line is chosen (see **Important** note above). If you have more than one lentiviral construct, we recommend that you titer all of the lentiviral constructs using the same mammalian cell line.

Antibiotic Selection

The pLenti6/R4R2/V5-DEST expression construct contains the Blasticidin resistance gene (*bsd*) (Kimura *et al.*, 1994) to allow for Blasticidin selection (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965) of mammalian cells that have stably transduced the lentiviral construct.

Note: Blasticidin is supplied with the ViraPower™ Promoterless Lentiviral Gateway® Expression System, but is also available separately from Invitrogen or as part of the ViraPower™ Lentiviral Support Kits (see page viii for ordering information).

Preparing Blasticidin

For more information about how to prepare and handle Blasticidin, refer to the **Appendix**, page 45.

Continued on next page

Titering Your Lentiviral Stock, Continued

Determining Antibiotic Sensitivity

Since you will be selecting for stably transduced cells using Blasticidin, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). Typically, concentrations ranging from 2–10 µg/ml Blasticidin are sufficient to kill most untransduced mammalian cell lines. We recommend that you test a range of concentrations (see protocol below) to ensure that you determine the minimum concentration necessary for your cell line.

1. Plate cells at approximately 25% confluence. Prepare a set of 6–7 plates. Allow cells to adhere overnight.
2. The next day, substitute culture medium with medium containing varying concentrations of Blasticidin.
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Determine the appropriate concentration of Blasticidin that kills the cells within 10–14 days after addition of antibiotic.

Using Polybrene® During Transduction

Transduction of lentivirus into mammalian cells may be enhanced if cells are transduced in the presence of hexadimethrine bromide (Polybrene®). For best results, we recommend performing transduction in the presence of Polybrene®. Note however, that some cells are sensitive to Polybrene® (*e.g.* primary neurons). Before performing any transduction experiments, you may want to test your cell line for sensitivity to Polybrene®. If your cells are sensitive to Polybrene® (*e.g.* exhibit toxicity or phenotypic changes), do not add Polybrene® during transduction. In this case, cells should still be successfully transduced.

Preparing and Storing Polybrene®

Follow the instructions below to prepare Polybrene® (Sigma, Catalog no. H9268):

1. Prepare a 6 mg/ml stock solution in deionized, sterile water.
2. Filter-sterilize and dispense 1 ml aliquots into sterile microcentrifuge tubes.
3. Store at –20°C for long-term storage. Stock solutions may be stored at –20°C for up to 1 year. Do not freeze/thaw the stock solution more than 3 times as this may result in loss of activity.

   Note: The working stock may be stored at 4°C for up to 2 weeks.

Continued on next page

Titering Your Lentiviral Stock, Continued

Materials Needed

You will need the following items:

- Your pLenti6/R4R2/V5-DEST lentiviral stock (store at –80°C until use)
- Your pLenti6/UbC/V5-GW/*lacZ* lentiviral stock (store at –80°C until use)
- Adherent mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/ml Polybrene®, if desired
- 6-well tissue culture plates
- Blasticidin (10 mg/ml stock) for selection
- Crystal violet (Sigma, Catalog no. C3886; prepare a 1% crystal violet solution in 10% ethanol)
- Phosphate-Buffered Saline (PBS)

Remember that you will be working with media containing infectious virus. Follow the recommended Federal and institutional guidelines for working with BL-2 organisms.

- Perform all manipulations within a certified biosafety cabinet.
- Treat media containing virus with bleach.
- Treat used pipets, pipette tips, and other tissue culture supplies with bleach and dispose of as biohazardous waste.
- Wear gloves, a laboratory coat, and safety glasses or goggles when handling viral stocks and media containing virus.

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Titering Your Lentiviral Stock, Continued

Transduction and Titering Procedure

Follow the procedure below to determine the titer of your lentiviral stock using the mammalian cell line of choice. You will use **at least** one 6-well plate for every lentiviral stock to be titered (one mock well plus five dilutions).

1. The day before transduction (Day 1), trypsinize and count the cells, plating them in a 6-well plate such that they will be 30–50% confluent at the time of transduction. Incubate cells at 37°C overnight.

   Example: When using HT1080 cells, we usually plate 2×10^5 cells per well in a 6-well plate.

2. On the day of transduction (Day 2), thaw your lentiviral stock and prepare 10-fold serial dilutions ranging from 10^{-2} to 10^{-6}. For each dilution, dilute the lentiviral stock into complete culture medium to a final volume of 1 ml. **DO NOT** vortex.

   Note: You may prepare a wider range of serial dilutions (10^{-2} to 10^{-8}), if desired.

3. Remove the culture medium from the cells. Mix each dilution gently by inversion and add to one well of cells (total volume = 1 ml).
4. Add Polybrene® (if desired) to each well to a final concentration of 6 µg/ml. Swirl the plate gently to mix. Incubate at 37°C overnight.
5. The following day (Day 3), remove the medium containing virus and replace with 2 ml of complete culture medium.
6. The following day (Day 4), remove the medium and replace with complete culture medium containing the appropriate amount of Blasticidin to select for stably transduced cells.
7. Replace medium with fresh medium containing Blasticidin every 3-4 days.
8. After 10-12 days of selection (day 14-16), you should see no live cells in the mock well and discrete Blasticidin-resistant colonies in one or more of the dilution wells. Remove the medium and wash the cells twice with PBS.
9. Add crystal violet solution (1 ml for 6-well dish; 5 ml for 10 cm plate) and incubate for 10 minutes at room temperature.
10. Remove the crystal violet stain and wash the cells with PBS. Repeat wash.
11. Count the blue-stained colonies and determine the titer of your lentiviral stock.

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Titering Your Lentiviral Stock, Continued

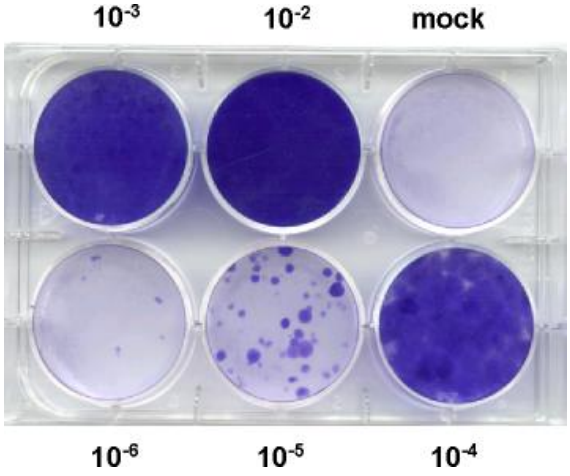
What You Should See

When titering pLenti lentiviral stocks using HT1080 cells, we generally obtain titers ranging from 5×10^5 to 2×10^7 transducing units (TU)/ml (see below for an example).

Note: If the titer of your lentiviral stock is less than 1×10^5 TU/ml, we recommend producing a new lentiviral stock. See page 30 and the **Troubleshooting** section, page 40 for more tips and guidelines to optimize your viral yield.

Example of Expected Results

In this experiment, a pLenti6 lentiviral stock was generated using the protocol on page 28. HT1080 cells were transduced with 10-fold serial dilutions of the lentiviral supernatant (10^{-2} to 10^{-6} dilutions) or untransduced (mock) following the protocol on page 34. Forty-eight hours post-transduction, the cells were placed under Blasticidin selection (10 µg/ml). After 10 days of selection, the cells were stained with crystal violet (see plate below), and colonies were counted.



In the plate above, the colony counts were:

- Mock: no colonies
- 10^{-2} dilution: confluent; undeterminable
- 10^{-3} dilution: confluent; undeterminable
- 10^{-4} dilution: confluent; undeterminable
- 10^{-5} dilution: 46
- 10^{-6} dilution: 5

Thus, the titer of this lentiviral stock is 4.8×10^6 TU/ml (*i.e.* average of 46×10^5 and 5×10^6).

Transduction of Mammalian Cells and Expression Analysis

Introduction

Once you have generated a lentiviral stock with a suitable titer, you are ready to transduce the lentiviral construct into the mammalian cell line of choice and assay for expression of your recombinant protein. Guidelines are provided below.

Reminder: Remember that your lentiviral construct contains a deletion in the 3′ LTR that leads to self-inactivation of the lentivirus after transduction into mammalian cells. Once integrated into the genome, the lentivirus can no longer produce packageable virus.

Transient vs. Stable Expression

After transducing your lentiviral construct into the mammalian cell line of choice, you may assay for expression of your gene of interest in the following ways:

- Pool a heterogeneous population of cells and test for expression directly after transduction (*i.e.* "transient" expression). Note that you must wait for a minimum of 48–72 hours after transduction before harvesting your cells to allow expressed protein to accumulate in transduced cells.
- Select for stably transduced cells using Blasticidin. This requires a minimum of 10–12 days after transduction, but allows generation of clonal cell lines that stably express the gene of interest.

  Note: We have observed stable expression of a target gene for at least 6 weeks following transduction and selection.

Determining Antibiotic Sensitivity for Your Cell Line

If you wish to select for stably transduced cells, you must first determine the minimum concentration of Blasticidin required to kill your untransduced mammalian cell line (*i.e.* perform a kill curve experiment). For guidelines to perform a kill curve experiment, see page 32. If you titered your lentiviral construct in the same mammalian cell line that you are using to perform your stable expression experiment, then you may use the same concentration of Blasticidin for selection that you used for titering.

Multiplicity of Infection (MOI)

To obtain optimal expression of your gene of interest, you will need to transduce the lentiviral construct into your mammalian cell line of choice using a suitable MOI. MOI is defined as the number of virus particles per cell and generally correlates with the number of integration events and as a result, expression. Typically, expression levels increase linearly as the MOI increases.

Determining the Optimal MOI

A number of factors can influence determination of an optimal MOI including the nature of your mammalian cell line (*e.g.* non-dividing vs. dividing cell type; see **Note** on the next page), its transduction efficiency, your application of interest, and the nature of your gene of interest. If you are transducing your lentiviral construct into the mammalian cell line of choice for the first time, we recommend using a range of MOIs (*e.g.* 0, 0.05, 0.1, 0.5, 1, 2, 5) to determine the MOI required to obtain optimal expression of your recombinant protein for your particular application.

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Note

In general, we have found that 80–90% of the cells in an actively dividing cell line (*e.g.* HT1080) express a target gene when transduced at an MOI of ~1. Some non-dividing cell types transduce lentiviral constructs less efficiently. For example, only about 50% of the cells in a culture of primary human fibroblasts express a target gene when transduced at an MOI of ~1. If you are transducing your lentiviral construct into a non-dividing cell type, you may need to increase the MOI to achieve optimal expression levels for your recombinant protein.

Positive Control

If you have generated the pLenti6/UbC/V5-GW/*lacZ* positive control lentiviral construct, we recommend using the lentiviral stock to help you determine the optimal MOI for your particular cell line and application. Once you have transduced the control lentivirus into your mammalian cell line of choice, β-galactosidase will be constitutively expressed and can be easily assayed (see page 39).

Important

Remember that viral supernatants are generated by harvesting spent media containing virus from the 293FT producer cells. Spent media lacks nutrients and may contain some toxic waste products. If you are using a large volume of viral supernatant to transduce your mammalian cell line (*e.g.* 1 ml of viral supernatant per well in a 6-well plate), note that growth characteristics or morphology of the cells may be affected during transduction. These effects are generally alleviated after transduction when the media is replaced with fresh, complete media.

Concentrating Virus

It is possible to concentrate VSV-G pseudotyped lentiviruses using a variety of methods without significantly affecting their transducibility. If the titer of your lentiviral stock is relatively low (less than 5×10^5 TU/ml) and your experiment requires that you use a large volume of viral supernatant (*e.g.* a relatively high MOI), you may wish to concentrate your virus before proceeding to transduction. For details and guidelines to concentrate your virus, refer to published reference sources (Yee, 1999).

Materials Needed

You will need the following items:

- Your titered lentiviral stock (store at –80°C until use)
- Mammalian cell line of choice
- Complete culture medium for your cell line
- 6 mg/ml Polybrene®, if desired
- Appropriately sized tissue culture plates for your application
- Blasticidin (if selecting for stably transduced cells)

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Transduction of Mammalian Cells and Expression Analysis, Continued

Transduction Procedure

Follow the procedure below to transduce the mammalian cell line of choice with your lentiviral construct.

1. Plate cells in complete media as appropriate for your application.
2. On the day of transduction (Day 1), thaw your lentiviral stock and dilute (if necessary) the appropriate amount of virus (at a suitable MOI) into fresh complete medium. Keep the total volume of medium containing virus as low as possible to maximize transduction efficiency. **DO NOT** vortex.
3. Remove the culture medium from the cells. Mix the medium containing virus gently by pipetting and add to the cells.
4. Add Polybrene® (if desired) to a final concentration of 6 µg/ml. Swirl the plate gently to mix. Incubate at 37°C overnight.

   Note: If you are transducing cells with undiluted viral stock and are concerned about possible toxicity or growth effects caused by overnight incubation, it is possible to incubate cells for as little as 6 hours prior to changing medium.
5. The following day (Day 2), remove the medium containing virus and replace with fresh, complete culture medium.
6. The following day (Day 3), perform one of the following:
   - Harvest the cells and assay for expression of your recombinant protein if you are performing transient expression experiments.
   - Remove the medium and replace with fresh, complete medium containing the appropriate amount of Blasticidin to select for stably transduced cells. Proceed to Step 7.
7. Replace medium with fresh medium containing Blasticidin every 3–4 days until antibiotic-resistant colonies can be identified (generally 10–12 days after selection).
8. Pick at least 5 Blasticidin-resistant colonies (see **Note** below) and expand each clone to assay for expression of the recombinant protein.

Note

Integration of the lentivirus into the genome is random. Depending upon the influence of the surrounding genomic sequences at the integration site, you may see varying levels of recombinant protein expression from different Blasticidin-resistant clones. We recommend testing at least 5 Blasticidin-resistant clones and selecting the clone that provides the optimal expression of your recombinant protein for further studies.

Detecting Recombinant Protein

You may use any method of choice to detect your recombinant protein of interest including functional analysis, immunofluorescence, or Western blot. If you have cloned your gene of interest in frame with the C-terminal V5 epitope tag, you may detect your recombinant protein in a Western blot using one of the Anti V5 Antibodies available from Invitrogen (see page viii for ordering information). For more information, visit our website at www.invitrogen.com or contact **Technical Support** (page 56).

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Assaying for β-galactosidase

If you transduce mammalian cells with the pLenti6/UbC/V5-GW/*lacZ* positive control lentivirus, you may assay for β-galactosidase expression by Western blot analysis or activity assay using cell-free lysates (Miller, 1972). Invitrogen offers the β-gal Antiserum and the β-Gal Assay Kit for fast and easy detection of β-galactosidase expression. See page ix for ordering information.

Note: The β-galactosidase protein expressed from the pLenti6/UbC/V5-GW/*lacZ* control lentiviral construct is fused to the V5 epitope and is approximately 121 kDa in size. If you are performing Western blot analysis, you may also use the Anti V5 Antibodies available from Invitrogen (see page viii for ordering information) for detection.

Troubleshooting

MultiSite Gateway® LR Reaction

The table below lists some potential problems and possible solutions that may help you troubleshoot the MultiSite Gateway® LR recombination reaction.

Problem	Cause	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing 100 µg/ml ampicillin.
	Recombination reaction was not treated with proteinase K	Treat reactions with proteinase K before transformation.
	Used incorrect *att* sites for the reaction	Use the appropriate entry clones (*i.e.* *att*L4 and *att*R1-flanked entry clone and *att*L1 and *att*L2-flanked entry clone) and pLenti6/R4R2/V5-DEST for the MultiSite Gateway® LR reaction (see page 11 for details about suitable entry vectors to use to generate entry clones).
	LR Clonase™ II Plus enzyme mix is inactive or didn't use suggested amount of LR Clonase™ II Plus enzyme mix	• Store the LR Clonase™ II Plus enzyme mix at –80°C for long term storage (> 6 months) • Do not freeze/thaw the LR Clonase™ II Plus enzyme mix more than 10 times. • Use the recommended amount of LR Clonase™ II Plus enzyme mix (see page 20).
	Used LR Clonase™ enzyme mix	Use the LR Clonase™ II Plus enzyme mix for the MultiSite Gateway® LR reaction. **Do not** use other LR Clonase™ enzyme mixes.
	Too much DNA was used in a MultiSite Gateway® LR reaction	Use an equimolar amount of each entry clone and destination vector. Do not exceed 1 µg of total DNA in the reaction.
	MultiSite Gateway® LR reaction not incubated for sufficient time	Incubate the MultiSite Gateway® LR reaction at 25°C for 16–24 hours
	Insufficient amount of *E. coli* transformed or plated	Transform 2 µl of the reaction; plate 50 µl or 100 µl.
	Did not perform the 1 hour grow-out period before plating the transformation mixture	After the heat-shock step, add S.O.C. Medium and incubate the transformation mixture for 1 hour at 37°C with shaking before plating.

Continued on next page

MultiSite Gateway® LR Reaction, continued

Problem	Cause	Solution
Different sized colonies (*i.e.* large and small) appear when using TOP10 or DH5α *E. coli* for transformation	Some transformants contain plasmids in which unwanted recombination has occurred between 5′ and 3′ LTRs	• Select for transformants on LB plates containing **both** 100 µg/ml ampicillin and 50 µg/ml Blasticidin. • Use the One Shot® Stbl3™ Chemically Competent *E. coli* supplied with the kit for transformation. Stbl3™ *E. coli* are recommended for cloning unstable DNA including lentiviral DNA containing direct repeats and generally give rise to fewer unwanted recombinants.
Few or no colonies obtained from the transformation control	Competent cells stored incorrectly	Store competent cells at –80°C.
	After addition of DNA, competent cells mixed by pipetting up and down	After adding the DNA, mix competent cells gently. **Do not** mix by pipetting up and down.

Generating the Lentiviral Stock

The table below lists some potential problems and possible solutions that may help you troubleshoot your cotransfection and titering experiments.

Problem	Cause	Solution
Low viral titer	Low transfection efficiency: • Used poor quality expression construct plasmid DNA (*i.e.* plasmid DNA from a mini-prep) • Unhealthy 293FT cells; cells exhibit low viability • Cells transfected in media containing antibiotics (*i.e.* Geneticin®) • Plasmid DNA:transfection reagent ratio incorrect • 293FT cells plated too sparsely	• **Do not** use mini-prep plasmid DNA for transfection. • Use healthy 293FT cells under passage 20; **do not overgrow**. • **Do not** add antibiotics to media during transfection as this reduces transfection efficiency and causes cell death. • Use a DNA (in µg):Lipofectamine™ 2000 (in µl) ratio ranging from 1:2 to 1:3. • Plate cells such that they are 90–95% confluent at the time of transfection **OR** use the recommended transfection protocol (*i.e.* add cells to media containing DNA-lipid complexes; see page 28).

Continued on next page

Generating the Lentiviral Stock, continued

Problem	Cause	Solution
Low viral titer, continued	Transfected cells not cultured in media containing sodium pyruvate	One day after transfection, remove media containing DNA-lipid complexes and replace with media containing sodium pyruvate. Sodium pyruvate provides an extra energy source for the cells.
	Viral supernatant harvested too early	Viral supernatants can generally be collected 48–72 hours posttransfection. If many cells are still attached to the plate and look healthy at this point, wait an additional 24 hours before harvesting the viral supernatant.
	Viral supernatant too dilute	Concentrate virus using any method of choice (Yee, 1999).
	Viral supernatant frozen and thawed multiple times	**Do not** freeze/thaw viral supernatant more than 3 times.
	Poor choice of titering cell line	Use HT1080 cells or another adherent cell line with the characteristics discussed on page 31.
	The size of the insert (promoter + gene) is large	Viral titers generally decrease as the size of the insert increases; inserts larger than 4.5–5 kb are not recommended.
	Gene of interest is toxic to cells	Do not generate constructs containing activated oncogenes or potentially harmful genes.
	Polybrene® not included during transduction	Transduce mammalian cells with the lentiviral construct in the presence of Polybrene®.
	Lipofectamine™ 2000 handled incorrectly	• Store at 4°C. **Do not freeze.** • Mix gently by inversion before use. **Do not vortex.**
No colonies obtained upon titering	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve experiment. Use the minimum Blasticidin concentration required to kill your untransduced cell line.
	Viral stocks stored incorrectly	Aliquot and store stocks at –80°C. Do not freeze/thaw more than 3 times.
	Polybrene® not included during transduction	Transduce mammalian cells with the lentiviral construct in the presence of Polybrene®.
Titer indeterminable; cells confluent	Too little Blasticidin used for selection	Increase amount of Blasticidin used for selection.
	Viral supernatant not diluted sufficiently	Titer lentivirus using a wider range of 10-fold serial dilutions (*e.g.* 10^{-2} to 10^{-8}).

Continued on next page

Transducing Mammalian Cells

The table below lists some potential problems and possible solutions that may help you troubleshoot your transduction and expression experiment.

Problem	Cause	Solution
No expression of the gene of interest	Promoter silencing	• If you are using a viral promoter to express the gene of interest, note that lentiviral constructs can integrate into a chromosomal region that down-regulates or silences the promoter. Screen multiple antibiotic-resistant clones and select the one with the highest expression levels. • Use a promoter that is not subject to silencing to express the gene of interest.
	Viral stocks stored incorrectly	Aliquot and store stocks at –80°C. Do not freeze/thaw more than 3 times.
Poor expression of the gene of interest	Low transduction efficiency: • Polybrene® not included during transduction • Non-dividing cell type used	• Transduce the lentiviral construct into cells in the presence of Polybrene®. • Transduce your lentiviral construct into cells using a higher MOI.
	MOI too low	Transduce your lentiviral construct into cells using a higher MOI.
	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve. Use the minimum Blasticidin concentration required to kill your untransduced cell line.
	Cells harvested too soon after transduction	Do not harvest cells until at least 48–72 hours after transduction to allow expressed protein to accumulate in transduced cells.
	Gene of interest is toxic to cells	Generating constructs containing activated oncogenes or potentially harmful genes is not recommended.
Cytotoxic effects observed after transduction	Large volume of viral supernatant used for transduction	• Remove the "spent" medium containing virus and replace with fresh, complete medium. • Concentrate the virus (Yee, 1999).
	Polybrene® used during transduction	Verify the sensitivity of your cells to Polybrene®. If cells are sensitive, omit the Polybrene® during transduction.
	Too much Blasticidin used for selection	Determine the Blasticidin sensitivity of your cell line by performing a kill curve. Use the minimum concentration of Blasticidin required to kill your untransduced cell line.
	Gene of interest is toxic to cells	Transduce cells at a lower MOI.

Appendix

Recipes

LB (Luria-Bertani) Medium

1.0% Tryptone
0.5% Yeast Extract
1.0% NaCl
pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.
2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C and add antibiotic, if desired.
4. Store at 4°C.

LB Plates Containing Ampicillin and Blasticidin

Follow the instructions below to prepare LB agar plates containing ampicillin and Blasticidin.

Important: The stability of Blasticidin may be affected by high temperature, therefore, **do not** add Blasticidin to warm LB agar. Let LB agar cool to room temperature before adding Blasticidin.

1. Prepare LB medium as above, but add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle for 20 minutes.
3. After autoclaving, cool to ~55°C, add ampicillin to a final concentration of 100 µg/ml and pour into 10 cm plates.
4. Let harden, then spread 50 µg/ml Blasticidin on each plate.
5. Invert and store at 4°C, in the dark. Plates containing Blasticidin may be stored at 4°C for up to 2 weeks.

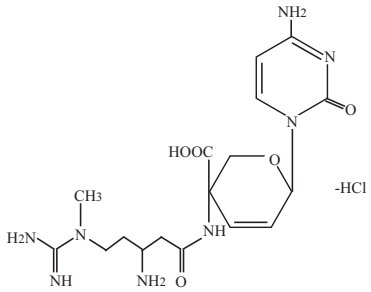
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al.*, 1958; Yamaguchi *et al.*, 1965). Resistance is conferred by expression of either one of two Blasticidin S deaminase genes: *bsd from Aspergillus terreus* (Kimura *et al.*, 1994) or *bsr* from *Bacillus cereus* (Izumi *et al.*, 1991). These deaminases convert Blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al.*, 1991).

Molecular Weight, Formula, and Structure

The formula for Blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of Blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (*e.g.* a laboratory coat) when handling Blasticidin. Weigh out Blasticidin and prepare solutions in a hood.

Preparing and Storing Stock Solutions

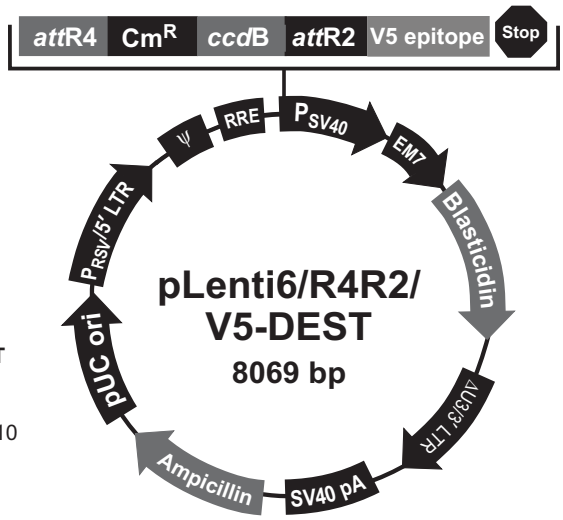
Blasticidin may be obtained separately from Invitrogen in 50 mg aliquots. Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5–10 mg/ml.

- Dissolve Blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at –20°C for long-term storage or store at 4°C for short-term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at –20°C.
- pH of the aqueous solution should be 7.0 to prevent inactivation of Blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and store the thawed stock solution at 4°C for up to 2 weeks.
- Medium containing Blasticidin may be stored at 4°C for up to 2 weeks.

Map of pLenti6/R4R2/V5-DEST

Map of pLenti6/R4R2/V5-DEST

The map below shows the elements of pLenti6/R4R2/V5-DEST. DNA from the entry clones replaces the region between bases 1829 and 3512. **The complete sequence for pLenti6/R4R2/V5-DEST is available from our website at www.invitrogen.com or by contacting Technical Support (page 56).**



Comments for pLenti6/R4R2/V5-DEST
8069 nucleotides

RSV/5′ LTR hybrid promoter: bases 1-410
RSV promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
*att*R4 site: bases 1823-1947
Chloramphenicol resistance gene (Cm^R): bases 2055-2714
*ccd*B gene: bases 3056-3361
*att*R2 site: bases 3402-3526
V5 epitope: bases 3579-3620
SV40 early promoter and origin: bases 3675-3983
EM7 promoter: bases 4038-4104
Blasticidin resistance gene: bases 4105-4503
ΔU3/3′ LTR: bases 4589-4823
ΔU3: bases 4589-4642
3′ LTR: bases 4643-4823
SV40 polyadenylation signal: bases 4895-5029
bla promoter: bases 5885-5983
Ampicillin (*bla*) resistance gene: bases 5984-6844
pUC origin: bases 6989-7662

Features of pLenti6/R4R2/V5-DEST

Features of the Vector

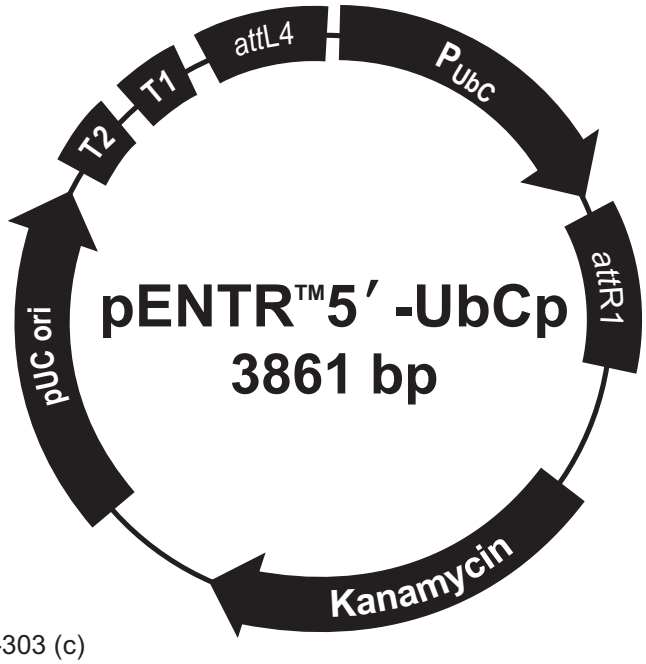
The pLenti6/R4R2/V5-DEST vector (8069 bp) contains the following elements. Features have been functionally tested and the vector fully sequenced.

Feature	Benefit
Rous Sarcoma Virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA (Dull *et al.*, 1998).
HIV-1 truncated 5′ LTR	Permits viral packaging and reverse transcription of the viral mRNA (Luciw, 1996).
5′ splice donor and 3′ acceptors	Enhances the biosafety of the vector by facilitating removal of the Ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev-dependent (Dull *et al.*, 1998).
HIV-1 psi (ψ) packaging signal	Allows viral packaging (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent nuclear export of unspliced viral mRNA (Kjems *et al.*, 1991; Malim *et al.*, 1989).
*att*R4 and *att*R2 sites	Bacteriophage λ-derived DNA recombination sequences that have been optimized to permit recombinational cloning of DNA fragments from multiple entry clones (Landy, 1989).
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
*ccd*B gene	Permits negative selection of the plasmid.
V5 epitope	Allows detection of the recombinant fusion protein by the Anti-V5 Antibodies (Southern *et al.*, 1991).
SV40 early promoter and origin	Allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen.
EM7 promoter	Synthetic prokaryotic promoter for expression of the selection marker in *E. coli.*
Blasticidin (*bsd*) resistance gene	Permits selection of stably transduced mammalian cell lines (Kimura *et al.*, 1994).
ΔU3/HIV-1 truncated 3′ LTR	Allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes (Dull *et al.*, 1998). The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 polyadenylation signal	Allows transcription termination and polyadenylation of mRNA.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase)	Allows selection of the plasmid in *E. coli*.
pUC origin	Permits high-copy replication and maintenance in *E. coli*.

Map of pENTR™5′/UbCp

Map of pENTR™5′/UbCp

pENTR™5′-UbCp is a 3861 bp entry construct containing the human UbC promoter (Hershko & Ciechanover, 1982; Schorpp *et al.*, 1996; Wulff *et al.*, 1990), and is included with the kit for use as a positive control in the MultiSite Gateway® LR recombination reaction. Note that *att*L4 and *att*R1 sites flank the UbC promoter. **The complete sequence of pENTR™5′-UbC is available from our website at www.invitrogen.com or by contacting Technical Support (page 56).**



Comments for pENTR™5′-UbCp
3861 nucleotides

*rrn*B T2 transcription terminator: bases 275-303 (c)
*rrn*B T1 transcription terminator: bases 437-480 (c)
M13 forward (-20) priming site: bases 546-561
*att*L4: bases 601-697
GW1 priming site: bases 639-663
Human UbC promoter: bases 698-1906
*att*R1: bases 1907-2030
GW3 priming site: bases 1937-1966
M13 reverse priming site: bases 2126-2142
Kanamycin resistance gene: 2255-3064
pUC origin: bases 3185-3858

(c) = complementary strand

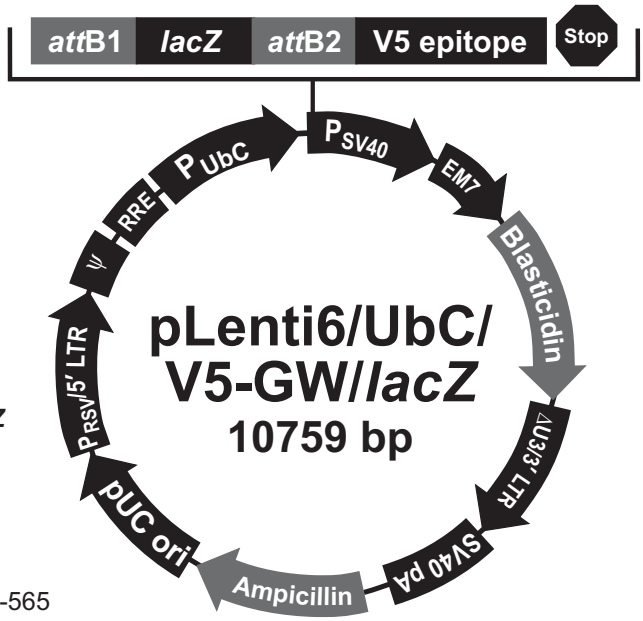
Map of pLenti6/UbC/V5-GW/*lacZ*

Description

pLenti6/UbC/V5-GW/*lacZ* is a 10759 bp control vector expressing β-galacto-sidase under the control of the UbC promoter, and is supplied with the kit for use as an expression control to help you optimize lentiviral production. The vector was generated using the Gateway® LR recombination reaction between an entry clone containing the *lacZ* gene and the pLenti6/UbC/V5-DEST vector. β-galactosidase is expressed as a C-terminal V5 fusion protein with a molecular weight of approximately 121 kDa.

Map of pLenti6/UbC/V5-GW/*lacZ*

The map below shows the elements of pLenti6/UbC/V5-GW/*lacZ*. **The complete sequence of the vector is available from our website at www.invitrogen.com or by contacting Technical Support (page 56).**



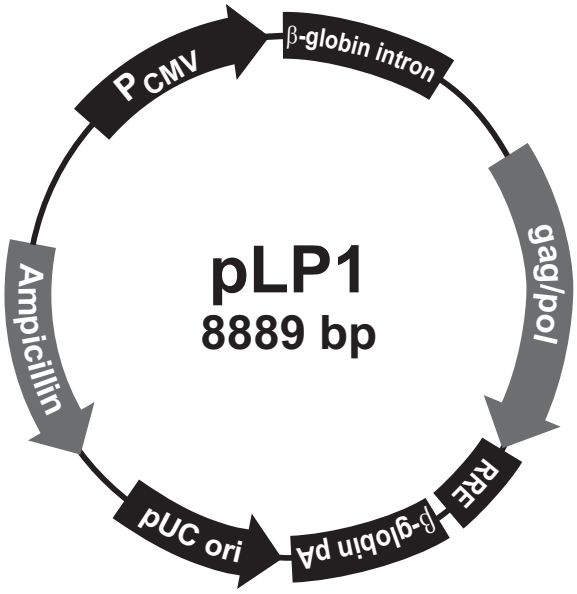
Comments for pLenti6/UbC/V5-GW/*lacZ*
10759 nucleotides

RSV enhancer/promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
UbC promoter: bases 1798-3016
*att*B1 site: bases 3072-3096
lacZ ORF: bases 3116-6172
*att*B2 site: bases 6192-6216
V5 epitope: bases 6269-6310
SV40 early promoter and origin: bases 6365-6673
EM7 promoter: bases 6728-6794
Blasticidin resistance gene: bases 6795-7193
ΔU3/HIV-1 3′ LTR: bases 7279-7513
    ΔU3: bases 7279-7332
    Truncated HIV-1 3′ LTR: bases 7333-7513
SV40 polyadenylation signal: bases 7585-7716
bla promoter: bases 8575-8673
Ampicillin (*bla*) resistance gene: bases 8674-9534
pUC origin: bases 9679-10352

Map of pLP1

pLP1 Map

The figure below shows the features of the pLP1 vector. Note that the *gag* and *pol* genes are initially expressed as a gag/pol fusion protein, which is then self-cleaved by the viral protease into individual Gag and Pol polyproteins. **The complete sequence of pLP1 is available for downloading from our website at www.invitrogen.com or by contacting Technical Support (page 56).**



Comments for pLP1
8889 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
HIV-1 gag/pol sequences: bases 1355-5661
gag coding sequence: bases 1355-2857
gag/pol frameshift: base 2650
pol coding sequence: bases 2650-5661
HIV-1 Rev response element (RRE): bases 5686-5919
Human β-globin polyadenylation signal: bases 6072-6837
pUC origin: bases 6995-7668 (C)
Ampicillin (*bla*) resistance gene: bases 7813-8673 (C)
bla promoter: bases 8674-8772 (C)

C=complementary strand

Features of pLP1

Features of pLP1

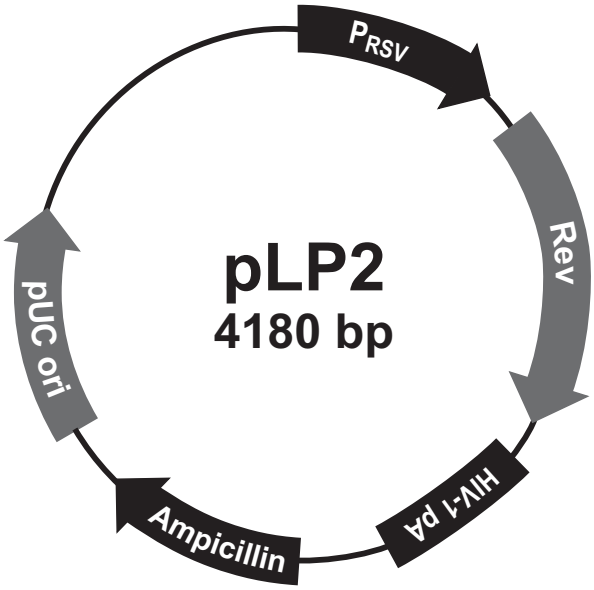
pLP1 (8889 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
Human cytomegalovirus (CMV) promoter	Permits high-level expression of the HIV-1 *gag* and *pol* genes in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the *gag* and *pol* genes in mammalian cells.
HIV-1 *gag* coding sequence	Encodes the viral core proteins required for forming the structure of the lentivirus (Luciw, 1996).
HIV-1 *pol* coding sequence	Encodes the viral replication enzymes required for replication and integration of the lentivirus (Luciw, 1996).
HIV-1 Rev response element (RRE)	Permits Rev-dependent expression of the *gag* and *pol* genes.
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

Map of pLP2

pLP2 Map

The figure below shows the features of the pLP2 vector. **The complete sequence of pLP2 is available for downloading from our website at www.invitrogen.com or by contacting Technical Support (page 56).**



Comments for pLP2
4180 nucleotides

RSV enhancer/promoter: bases 1-271
TATA box: bases 200-207
Transcription initiation site: base 229
RSV UTR: bases 230-271
HIV-1 Rev ORF: bases 391-741
HIV-1 LTR polyadenylation signal: bases 850-971
bla promoter: bases 1916-2014
Ampicillin (*bla*) resistance gene: bases 2015-2875
pUC origin: bases 3020-3693

Features of pLP2

Features of pLP2

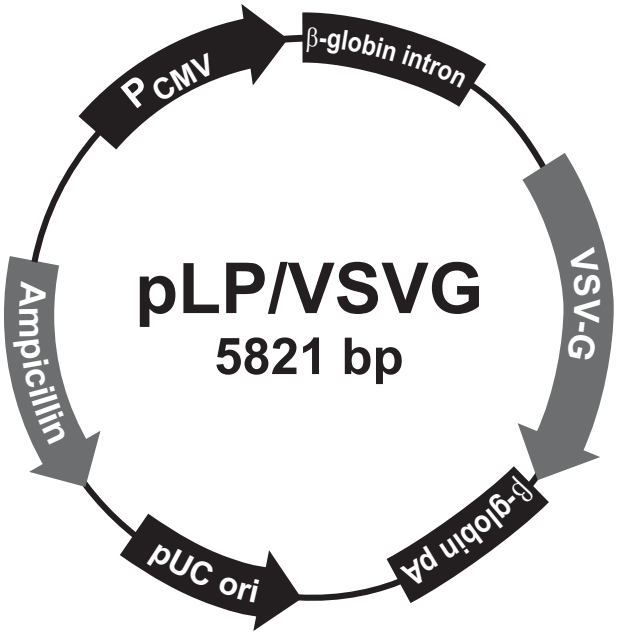
pLP2 (4180 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
RSV enhancer/promoter	Permits high-level expression of the *rev* gene (Gorman *et al.*, 1982).
HIV-1 Rev ORF	Encodes the Rev protein, which interacts with the RRE on pLP1 to induce Gag and Pol expression, and on the pLenti6/V5 expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles.
HIV-1 LTR polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.

Map of pLP/VSVG

pLP/VSVG Map

The figure below shows the features of the pLP/VSVG vector. **The complete sequence of pLP/VSVG is available for downloading from our website at www.invitrogen.com or by contacting Technical Support (page 56).**



Comments for pLP/VSVG
5821 nucleotides

CMV promoter: bases 1-747
TATA box: bases 648-651
Human β-globin intron: bases 880-1320
VSV G glycoprotein (VSV-G): bases 1346-2881
Human β-globin polyadenylation signal: bases 3004-3769
pUC origin: bases 3927-4600 (C)
Ampicillin (*bla*) resistance gene: bases 4745-5605 (C)
bla promoter: bases 5606-5704 (C)

C=complementary strand

Features of pLP/VSVG

Features of pLP/VSVG

pLP/VSVG (5821 bp) contains the following elements. Features have been functionally tested.

Feature	Benefit
Human CMV promoter	Permits high-level expression of the VSV-G gene in mammalian cells (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987).
Human β-globin intron	Enhances expression of the VSV-G gene in mammalian cells.
VSV G glycoprotein (VSV-G)	Encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of a pseudotyped retrovirus with a broad host range (Burns *et al.*, 1993; Emi *et al.*, 1991; Yee *et al.*, 1994).
Human β-globin polyadenylation signal	Allows efficient transcription termination and polyadenylation of mRNA.
pUC origin of replication (*ori*)	Permits high-copy replication and maintenance in *E. coli*.
Ampicillin (*bla*) resistance gene	Allows selection of the plasmid in *E. coli*.

Technical Support

Web Resources

Visit the Invitrogen website at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information.
- Access to the Invitrogen Online Catalog.
- Additional product information and special offers.

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MSDS

Material Safety Data Sheets (MSDSs) are available at www.invitrogen.com/msds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product. The CofA is available at www.invitrogen.com/support, and is searchable by product lot number, which is printed on each box.

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Purchaser Notification

Introduction

Use of the ViraPower™ Promoterless Lentiviral Gateway® Kits is covered under a number of different licenses including those detailed below.

Information for European Customers

The 293FT Cell Line is genetically modified and carries the pUC-derived plasmid, pCMVSPORT6Tag.neo. As a condition of sale, use of this product must be in accordance with all applicable local legislation and guidelines including EC Directive 90/219/EEC on the contained use of genetically modified organisms.

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Gateway® Clone Distribution Policy

For additional information about Invitrogen's policy for the use and distribution of Gateway® clones, see the section entitled **Gateway® Clone Distribution Policy**, page 61.

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Purchaser Notification, Continued

Limited Use Label License No. 27: RNA Transfection	Use of this product in conjunction with methods for the introduction of RNA molecules into cells may require licenses to one or more patents or patent applications. Users of these products should determine if any licenses are required.
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Limited Use Label License No. 109: Retroviral Helper Lines	Retroviral helper cell lines are licensed from Wisconsin Alumni Research Foundation, under U.S. Patents and corresponding patents and applications in other countries for internal research purposes only. Use of these cell lines for Commercial Purposes requires a license from Life Technologies.

Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Invitrogen's policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Invitrogen's commercially available Gateway® Technology.

Gateway® Entry Clones

Invitrogen understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Invitrogen.

Gateway® Expression Clones

Invitrogen also understands that Gateway® expression clones, containing *att*B1 and *att*B2 sites, may be generated by academic and government researchers for the purpose of scientific research. Invitrogen agrees that such clones may be distributed for scientific research by academic and government organizations without royalty payment to Invitrogen. Organizations other than academia and government may also distribute such Gateway® expression clones for a nominal fee ($10 per clone) payable to Invitrogen.

Additional Terms and Conditions

We would ask that such distributors of Gateway® entry and expression clones indicate that such clones may be used only for research purposes, that such clones incorporate the Gateway® Technology, and that the purchase of Gateway® Clonase™ from Invitrogen is required for carrying out the Gateway® recombinational cloning reaction. This should allow researchers to readily identify Gateway® containing clones and facilitate their use of this powerful technology in their research. Use of Invitrogen's Gateway® Technology, including Gateway® clones, for purposes other than scientific research may require a license and questions concerning such commercial use should be directed to Invitrogen's licensing department at 760-603-7200.

References

Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989) Cloning, Structure, and Expression of the Mitochondrial Cytochrome P-450 Sterol 26-Hydroxylase, a Bile Acid Biosynthetic Enzyme. J. Biol. Chem. *264*, 8222-8229

Baek, S. C., Lin, Q., Robbins, P. B., Fan, H., and Khavari, P. A. (2001) Sustainable Systemic Delivery via a Single Injection of Lentivirus into Human Skin Tissue. Hum. Gene Ther. *12*, 1551-1558

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985) A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell *41*, 521-530

Buchschacher, G. L., Jr., and Wong-Staal, F. (2000) Development of Lentiviral Vectors for Gene Therapy for Human Diseases. Blood *95*, 2499-2504

Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J.-K. (1993) Vesicular Stomatitis Virus G Glycoprotein Pseudotyped Retroviral Vectors: Concentration to a Very High Titer and Efficient Gene Transfer into Mammalian and Nonmammalian Cells. Proc. Natl. Acad. Sci. USA *90*, 8033-8037

Ciccarone, V., Chu, Y., Schifferli, K., Pichet, J.-P., Hawley-Nelson, P., Evans, K., Roy, L., and Bennett, S. (1999) Lipofectamine™ 2000 Reagent for Rapid, Efficient Transfection of Eukaryotic Cells. Focus *21*, 54-55

Dull, T., Zufferey, R., Kelly, M., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998) A Third-Generation Lentivirus Vector with a Conditional Packaging System. J. Virol. *72*, 8463-8471

Emi, N., Friedmann, T., and Yee, J.-K. (1991) Pseudotype Formation of Murine Leukemia Virus with the G Protein of Vesicular Stomatitis Virus. J. Virol. *65*, 1202-1207

Gallichan, W. S., Kafri, T., Krahl, T., Verma, I. M., and Sarvetnick, N. (1998) Lentivirus-mediated Transduction of Islet Grafts with Interleukin 4 Results in Sustained Gene Expression and Protection from Insulitis. Human Gene Therapy *9*, 2717-2726

Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I., and Howard, B. H. (1982) The Rous Sarcoma Virus Long Terminal Repeat is a Strong Promoter When Introduced into a Variety of Eukaryotic Cells by DNA-mediated Transfection. Proc. Natl. Acad. Sci. USA *79*, 6777-6781

Hartley, J. L., Temple, G. F., and Brasch, M. A. (2000) DNA Cloning Using *in vitro* Site-Specific Recombination. Genome Research *10*, 1788-1795

Hershko, A., and Ciechanover, A. (1982) Mechanisms of Intracellular Protein Breakdown. Ann. Rev. Biochem. *51*, 335-364

Izumi, M., Miyazawa, H., Kamakura, T., Yamaguchi, I., Endo, T., and Hanaoka, F. (1991) Blasticidin S-Resistance Gene (*bsr*): A Novel Selectable Marker for Mammalian Cells. Exp. Cell Res. *197*, 229-233

Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997) Sustained Expression of Genes Delivered Directly into Liver and Muscle by Lentiviral Vectors. Nature Genetics *17*, 314-317

Kimura, M., Takatsuki, A., and Yamaguchi, I. (1994) Blasticidin S Deaminase Gene from *Aspergillus terreus* (*BSD*): A New Drug Resistance Gene for Transfection of Mammalian Cells. Biochim. Biophys. ACTA *1219*, 653-659

Kjems, J., Brown, M., Chang, D. D., and Sharp, P. A. (1991) Structural Analysis of the Interaction Between the Human Immunodeficiency Virus Rev Protein and the Rev Response Element. Proc. Natl. Acad. Sci. USA *88*, 683-687

Kozak, M. (1987) An Analysis of 5´-Noncoding Sequences from 699 Vertebrate Messenger RNAs. Nucleic Acids Res. *15*, 8125-8148

Continued on next page

References, Continued

Kozak, M. (1990) Downstream Secondary Structure Facilitates Recognition of Initiator Codons by Eukaryotic Ribosomes. Proc. Natl. Acad. Sci. USA *87*, 8301-8305

Kozak, M. (1991) An Analysis of Vertebrate mRNA Sequences: Intimations of Translational Control. J. Cell Biology *115*, 887-903

Landy, A. (1989) Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. Ann. Rev. Biochem. *58*, 913-949

Lewis, P. F., and Emerman, M. (1994) Passage Through Mitosis is Required for Oncoretroviruses but not for the Human Immunodeficiency Virus. J. Virol. *68*, 510-516

Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002) Germline Transmission and Tissue-Specific Expression of Transgenes Delivered by Lentiviral Vectors. Science *295*, 868-872

Luciw, P. A. (1996) in *Fields Virology* (Fields, B. N., Knipe, D. M., Howley, P. M., Chanock, R. M., Melnick, J. L., Monath, T. P., Roizman, B., and Straus, S. E., eds), 3rd Ed., pp. 1881-1975, Lippincott-Raven Publishers, Philadelphia, PA

Malim, M. H., Hauber, J., Le, S. Y., Maizel, J. V., and Cullen, B. R. (1989) The HIV-1 Rev Trans-activator Acts Through a Structured Target Sequence to Activate Nuclear Export of Unspliced Viral mRNA. Nature *338*, 254-257

Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Miyoshi, H., Takahashi, M., Gage, F. H., and Verma, I. M. (1997) Stable and Efficient Gene Transfer into the Retina Using an HIV-based Lentiviral Vector. Proc. Natl. Acad. Sci. USA *94*, 10319-10323

Naldini, L. (1998) Lentiviruses as Gene Transfer Agents for Delivery to Non-dividing Cells. Curr. Opin. Biotechnol. *9*, 457-463

Naldini, L. (1999) in *The Development of Human Gene Therapy* (Friedmann, T., ed), pp. 47-60, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996) Efficient Transfer, Integration, and Sustained Long-Term Expression of the Transgene in Adult Rat Brains Injected with a Lentiviral Vector. Proc. Natl. Acad. Sci. USA *93*, 11382-11388

Nelson, J. A., Reynolds-Kohler, C., and Smith, B. A. (1987) Negative and Positive Regulation by a Short Segment in the 5′-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Molec. Cell. Biol. *7*, 4125-4129

Park, F., and Kay, M. A. (2001) Modified HIV-1 Based Lentiviral Vectors Have an Effect on Viral Transduction Efficiency and Gene Expression *In Vitro* and *In Vivo*. Mol. Ther. *4*, 164-173

Peng, K. W., Pham, L., Ye, H., Zufferey, R., Trono, D., Cosset, F. L., and Russell, S. J. (2001) Organ Distribution of Gene Expression After Intravenous Infusion of Targeted and Untargeted Lentiviral Vectors. Gene Ther. *8*, 1456-1463

Pfeifer, A., Brandon, E. P., Kootstra, N., Gage, F. H., and Verma, I. M. (2001) Delivery of the Cre Recombinase by a Self-deleting Lentiviral Vector: Efficient Gene Targeting *In Vivo*. Proc. Natl. Acad. Sci. USA *98*, 11450-11455

Pfeifer, A., Kessler, T., Yang, M., Baranov, E., Kootstra, N., Cheresh, D. A., Hoffman, R. M., and Verma, I. M. (2001) Transduction of Liver Cells by Lentiviral Vectors: Analysis in Living Animals by Fluorescence Imaging. Mol. Ther. *3*, 319-322

Schorpp, M., Jäger, R., Schellander, K., Schenkel, J., Wagner, E. F., Weiher, H., and Angel, P. (1996) The Human Ubiquitin C Promoter Directs High Ubiquitous Expression of Transgenes in Mice. Nuc. Acids Res. *24*, 1787-1788

Continued on next page

References, Continued

Southern, J. A., Young, D. F., Heaney, F., Baumgartner, W., and Randall, R. E. (1991) Identification of an Epitope on the P and V Proteins of Simian Virus 5 That Distinguishes Between Two Isolates with Different Biological Characteristics. J. Gen. Virol. *72*, 1551-1557

Takahashi, M., Miyoshi, H., Verma, I. M., and Gage, F. H. (1999) Rescue from Photoreceptor Degeneration in the rd Mouse by Human Immunodeficiency Virus Vector-Mediated Gene Transfer. J. Virol. *73*, 7812-7816

Takeuchi, S., Hirayama, K., Ueda, K., Sakai, H., and Yonehara, H. (1958) Blasticidin S, A New Antibiotic. The Journal of Antibiotics, Series A *11*, 1-5

Wulff, B. S., O'Hare, M. M., Boel, E., Theill, L. E., and Schwartz, T. W. (1990) Partial Processing of the Neuropeptide Y Precursor in Transfected CHO Cells. FEBS Lett. *261*, 101-105

Yamaguchi, H., Yamamoto, C., and Tanaka, N. (1965) Inhibition of Protein Synthesis by Blasticidin S. I. Studies with Cell-free Systems from Bacterial and Mammalian Cells. J. Biochem (Tokyo) *57*, 667-677

Yee, J.-K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C., and Friedmann, T. (1994) A General Method for the Generation of High-Titer, Pantropic Retroviral Vectors: Highly Efficient Infection of Primary Hepatocytes. Proc. Natl. Acad. Sci. USA *91*, 9564-9568

Yee, J. K. (1999) in *The Development of Human Gene Therapy* (Friedmann, T., ed), pp. 21-45, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Yee, J. K., Moores, J. C., Jolly, D. J., Wolff, J. A., Respess, J. G., and Friedmann, T. (1987) Gene Expression from Transcriptionally Disabled Retroviral Vectors. Proc. Natl. Acad. Sci. USA *84*, 5197-5201

Yu, S. F., Ruden, T. v., Kantoff, P. W., Garber, C., Seiberg, M., Ruther, U., Anderson, W. F., Wagner, E. F., and Gilboa, E. (1986) Self-Inactivating Retroviral Vectors Designed for Transfer of Whole Genes into Mammalian Cells. Proc. Natl. Acad. Sci. USA *83*, 3194-3198

Zufferey, R., Dull, T., Mandel, R. J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998) Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. J. Virol. 72. 9873-9880

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Notes

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